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or in logarithmic form

$$p_aH = pK_1 - pf_a + \log \frac{[\text{HCO}_3^-]}{[\text{CO}_2]} = pK'_1 + \log \frac{[\text{HCO}_3^-]}{[\text{CO}_2]}$$

Addition of salt to the solution will lower the activity coefficient of the bicarbonate ions; K'_1 , therefore, will be greater and pK'_1 will be less; the acid will appear stronger. Addition of hemoglobin to the solution apparently works in the same direction (Van Slyke and collaborators, Stadie and Hawes (1928), Henriques (1929)), but the hemoglobin molecules apparently increase K'_1 (diminish pK'_1) much more than can be reckoned from the general laws of activity. Moreover the hemoglobin molecules should "depress the activity of the bicarbonate ions" in quite another way than do ordinary ions. According to the Debye-Hückel theory (and as experimentally verified by Hastings and Sendroy and by Stadie and Hawes (1928)) the following relation holds

$$pf_a = K \sqrt{\mu}$$

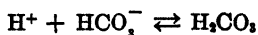
(μ = ionic strength), while an addition of hemoglobin to the solution according to the important work of Stadie and Hawes will act upon pf_a as follows

$$pf_a = K \cdot C_{\text{Hb}}$$

In other words, the hemoglobin molecules will behave towards the carbonic acid compounds not like a simple electrolyte or amphion (*cf.* Bjerrum (1923) and Rørdam (1925)) proportional to the square root of its concentration but as if it entered into combination with one of the carbonic acid compounds proportional to its concentration.

In what direction will alteration of the velocity of the reaction between the carbonic acid components act?

Van Slyke and Hawkins believe that hemoglobin (or cell contents) will accelerate the reaction



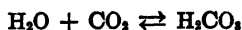
Most chemists, however, agree in supposing these reactions to be very rapid, and that they cannot be accelerated (see for example

TABLE I
Velocities at Which Reactions $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ Will Reach Equilibrium at pH 7.45
 Calculated from Faurholt (1924-25).

°C.	$\frac{1}{T} \cdot 10^{-3}$	$-\log K$	K	t when amount transformed is equal to									
				0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	
				sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	
0	3.663	2.796	0.0016	28.7	60.6	96.9	138.7	188.1	248.7	326.9	436.9	625.0	
18	3.436	1.900	0.0126	3.7	7.7	12.3	17.6	23.8	31.6	41.5	55.5	79.3	
25	3.355	1.58	0.0263	1.7	3.7	5.8	8.4	11.4	15.1	19.9	26.6	38.0	
30	3.330	1.38	0.0417	1.1	2.3	3.7	5.3	7.2	9.5	12.5	16.7	23.9	
35	3.246	1.18	0.0661	0.7	1.4	2.3	3.3	4.5	6.0	7.9	10.6	15.1	
40	3.195	1.0	0.1	0.46	0.97	1.55	2.22	3.01	3.98	5.23	6.99	10.0	

Faurholt (1924-25)); further acceleration of these velocities therefore does not apply to our problem.

In previous publications (1928, 1929) Henriques discussed the possibility of a catalytic acceleration of the processes



As shown by several investigators (see Faurholt for literature) these reactions are slow at neutral reaction.

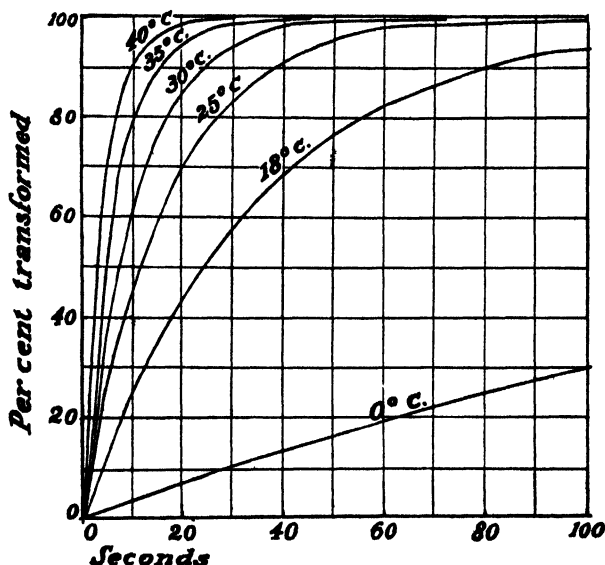


FIG. 1. The velocities at which the reactions $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$ will reach equilibrium at pH 7.45. The ordinates show the per cent of equilibrium amount transformed; the abscissæ, the time in seconds.

The data presented in Table I and Fig. 1 are calculated from Faurholt's data; the values for temperatures over 18° are extrapolated by the van't Hoff equation:

$$-\log K = A \cdot \frac{1}{T} + B$$

where K = reaction constant, and A and B are constants.

Henriques discarded the hypothesis of a catalytic acceleration of the hydration \rightleftharpoons dehydration because the mechanism of ac-

celerated CO₂ escape only affects a part of the combined CO₂ present (see the curves of Henriques and of Van Slyke and Hawkins); according to experience of catalytic reactions one would expect a catalytic acceleration of the dehydration throughout the whole course of the CO₂ escape, while it is quite improbable that any catalytic reaction should only comprise a lesser amount of the same compound (HCO₃⁻) and in proportion to the amount of hemoglobin (see Henriques' experiments at 18° (1929)).

It appears from Table I and Fig. 1 that the velocities of the hydration of CO₂ and dehydration of H₂CO₃ at physiological pH and temperatures higher than 30° are so great that it is impossible with "explosion" methods such as those of Henriques or Van Slyke and Hawkins to measure an acceleration with any exactitude. Henriques, therefore, carried out all gas equilibrations and velocity measurements at 18° and in the experiments with simple CO₂-bicarbonate solutions, as serum, found that the mechanical circumstances of his technique retarded the gas liberation from the solution to such an extent that the velocity constant was found to be only a third of that of Faurholt. Henriques concluded that experiments of this sort cannot give more than relative values. As I have been misunderstood on this point, I shall take the opportunity to express as my opinion that all explosion methods will show a systematic error dependent upon the dimensions of the apparatus, the time required for opening cocks, etc.; measurements with one apparatus, therefore, cannot be directly compared with measurements with another apparatus. But with the same apparatus and with an exact and uniform technique in working one person can obtain values that are comparable when the experiments are carried out at 18°.

Van Slyke and Hawkins made their gas equilibrium determinations at 38° and the reading of pressures in the constant volume apparatus at 23–25°.

As it appears from Table I and Fig. 1, the influence of the temperature on the reaction velocity is very important and the temperature must be kept constant throughout the whole experiment, during the gas equilibrations as well as the velocity measurements.

But at a temperature higher than 20° the experimental error will be appreciable and at temperatures higher than 25° the re-

action velocity of dehydration is so great that the error will be very important.'

Van Slyke and Hawkins find in one experiment with their technique that a minimal concentration of hemoglobin accelerates the escape of CO_2 quite as well as a high concentration. Henriques found a fairly good parallelism between concentration of hemoglobin and the amount of CO_2 evolved instantaneously in four experiments at 18° with hemoglobin concentrations of 14, 17, 21, and 24 per cent.

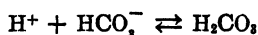
How is this disagreement between Henriques and Van Slyke and Hawkins to be explained?

First, some words concerning the technique. Originally Henriques used a method almost like that of Van Slyke and Hawkins but soon gave it up for two reasons: (1) the shortest possible reading time was 10 seconds; (2) an appreciable amount of the viscous hemoglobin solution remained as an adherent film in the evacuation chamber after the solution had been withdrawn. Henriques, therefore, constructed the two chamber apparatus, described in previous publications, where the whole amount of the hemoglobin solution (2 cc.) enters the 100 cc. large explosion chamber from the measuring cock and is broken into minute particles, in a fraction of a second, by smashing against the opposite wall; the gas analysis is made in the second solution-free chamber and the first gas sample for analysis can easily be taken after 5 seconds.

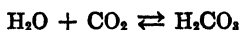
Secondly, some remarks on the velocity of the escape of CO_2 from CO_2 -supersaturated solutions in general. In common CO_2 gas analysis, as for instance by the methods of Van Slyke, the bound CO_2 is liberated by addition of stronger acid; the free CO_2 supersaturates the solution, forms bubbles, and escapes. The bubbling can be accelerated by several means; in gas analysis shaking is generally used as in Van Slyke's methods, and Van Slyke recommends 2 minutes shaking for the complete bubbling off of the CO_2 ; from soda water the bubbling can be accelerated by addition of some solid NaCl or by dipping a wooden pin into the fluid. The escape of CO_2 from a supersaturated solution by simple diffusion from the bulk of the fluid, through the surface, to the gas phase is a very slow one; the bubble formation in the bulk of the fluid accelerates the escape very much.

Thirdly, if a reaction consists of several reactions (either physical or chemical), the velocity of the original will, according to Nernst's law, be governed by the velocity of the slowest reaction (or reactions). At a temperature of 18° carbonic acid dehydration is comparatively slow (14 per cent of the equilibrium amount in 5 seconds), and the velocity of the CO₂ escape in an explosion analysis will be governed by the velocity of the dehydration. At a temperature of 35° carbonic acid dehydration is comparatively rapid (54 per cent of the equilibrium amount in 5 seconds), and the velocity of the CO₂ escape in the Van Slyke and Hawkins experiments will be governed by the velocity of the physical escape of CO₂ from the supersaturated solution. If some hemoglobin is added to the solution the physical escape of CO₂ is accelerated and even very small amounts of hemoglobin will produce a marked influence.

Let us assume, however, that Van Slyke and Hawkins were right in their interpretation that the rapid exchange of CO₂ from hemoglobin solutions is due to a catalytic acceleration, not of the process



but of the process



If the velocity of the dehydration of H₂CO₃ were accelerated, it would mean that the fraction in Equation 1 would be diminished and consequently K_{hydrate} would decrease, and, according to Equation 4, K_1 would *decrease*. An addition of hemoglobin to the solution, however, *increases* K_1 very appreciably; in fact, to such an extent and in such a way that we cannot interpret the increase as an activity phenomenon. If, therefore, Van Slyke and Hawkins should be right in their hypothesis, a still greater catalytic acceleration of the opposite reaction, the hydration of CO₂, would be demanded so that the fraction in Equation 1 would increase, and accordingly K_{hydrate} and K_1 would increase, as K_1 is experimentally shown to do.

But even that would not suffice.

For the extraordinary decrease of pK'_1 (increase of K'_1) by addition of hemoglobin is proportional to the *concentration* of hemoglobin and *not to the square root of its concentration* and the accelerating effect comprises only a part of the combined CO_2 present. Both questions are, however, interpreted by Henriques' hypothesis that a part of the combined CO_2 present is in a complex hemoglobin compound with an instantaneous reaction velocity in a way analogous to the instantaneously reacting carbaminic acids and alkyl carbonic acids.

A theoretical disagreement between Van Slyke and Henriques seems further to concern the conception of proteins as ampholytes (Van Slyke and Hawkins (1930) p. 277, last paragraph). Van Slyke holds the old conception, especially presented by Michaelis (1914) and Loeb (1922), while Henriques argues from the conception of Bjerrum (1923). Readers must be referred to Henriques' paper (1929).

Van Slyke and Hawkins (and Van Slyke in a private communication) attach much importance to the apparent inconsistency between Henriques' carbohemoglobin hypothesis and the extended titration experiments with hemoglobin, carried out in Van Slyke's laboratory with CO_2 (Van Slyke, Hastings, Heidelberger, and Neill, 1922; Hastings, Van Slyke, Neill, Heidelberger, and Harington, 1924) or with HCl (Hastings, Sendroy, Murray, and Heidelberger, 1924).

The calculation of the distribution ratios of the carbonic acid components from the many CO_2 titration experiments took place, however, before the year 1925; that is to say, before Van Slyke and collaborators as the pioneers found that hemoglobin "depresses the pK'_1 of CO_2 " to a much greater degree than can be reckoned from the laws of activity. It is not possible today for the present writer to recalculate the ratios. Instead of this, attention is drawn to the following facts.

Faurholt has, from his experimental data, calculated the strength of simpler carbaminic acids to $K = \text{about } 10^{-7}$; that is, the same order of magnitude as the apparent dissociation constant of CO_2 . It seems likely that a protein-carbaminic acid as the supposed carbohemoglobin acid should be of about the same strength.

Suppose, now, that a hemoglobin solution is titrated with CO_2

(volumetrically and electrometrically) and that a greater or lesser quantity of CO₂ is complex bound to hemoglobin. Three possibilities may be considered:

1. pK' of carbohemoglobin acid = pK' of CO₂
2. " " " " > " " "
3. " " " " < " " "

In the case of (1), in titrating no difference can be detected between the hemoglobin solution and a simple buffer solution of equal strength.

For (2), in titrating the CO₂ will appear as a weaker acid and the pK will increase in proportion to the concentration of hemoglobin.

For (3), in titrating the CO₂ will appear as a stronger acid and the pK will decrease in proportion to the concentration of hemoglobin.

From 1916, when Hasselbalch published his CO₂ titration method, until 1925, when Van Slyke and his collaborators demonstrated the effect of hemoglobin upon the dissociation constant of CO₂, the technique for these difficult experiments had not been sufficiently elaborated. All investigators, therefore, believed that the dissociation constant of CO₂ in the aqueous phase of hemoglobin solutions was the same as in simple aqueous solutions of the same ionic strength, and the distribution ratios of the carbonic acid components were calculated according to this.

Since the paper of Van Slyke, Hastings, Murray, and Sendroy (1925), work on CO₂-hemoglobin equilibrium has been published by Stadie and Hawes (1928) and by Henriques (1928, 1929).

It has been mentioned above that Stadie and Hawes in their CO₂ titration experiments found that hemoglobin "depresses pK_1' of the CO₂" very much and in proportion to the concentration of hemoglobin. On the assumptions made, these findings indicate that the possibility in (3) is the right one: an appreciable part of the CO₂ present is bound in a molecular union to hemoglobin, forming a complex acid which is stronger than CO₂.

The Donnan experiments of Henriques (carried out in 1924-25 and published in 1928 and 1929) can only be explained in the same way as those of Stadie and Hawes.

At the present stage of investigation, therefore, Henriques' hypothesis seems the most probable. Further experiments carried out with more suitable methods must decide the many questions involved.

Addendum—The appearance of these pages has been delayed owing to illness. In the meantime the last words of this paper seem to have been anticipated. Dirken and Mook (Dirken, M. N. J., and Mook, H. W., *J. Physiol.*, **70**, 373 (1930)) have published some remarkable experiments on the velocity of the binding of CO₂ to hemoglobin solutions. They have made use of the rational principle of Hartridge and Roughton and if they succeed in measuring the velocity of both reactions, the "explosion" method of Henriques and of Van Slyke and Hawkins will be only of historic interest.

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STUDIES OF BROMIDE DISTRIBUTION IN THE BLOOD

I. IN VITRO EXPERIMENTS OF BROMIDE AND CHLORIDE DISTRIBUTIONS

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Studies on the distribution of ions between cells and their environment have, in the past, dealt principally with the naturally occurring ions. Discrepancies which have been observed between the distribution of bicarbonate and chloride ions, have directed attention again to the factors other than the Gibbs-Donnan distribution law which might play a rôle in the distribution of diffusible ions. Since pharmacological literature contains references to the ability of bromides to *displace* chlorides from the body (1), and, on the other hand, since the chemical similarity of bromide and chloride ions is marked, it seemed to the authors that a study of the distribution of bromides and chlorides between serum and red blood corpuscles would provide an opportunity of further testing the general applicability of the Donnan law to ions not normally occurring. It was also hoped that such a study might throw some light upon the pharmacology of bromides.

It will be shown in the succeeding paper that although factors which alter the concentration of non-diffusible cell ions, such as changing cell pH and degree of oxygenation of the hemoglobin, lead to changes in the distribution of both bromides and chloride ions in a manner consistent with that demanded by the Donnan law, the absolute values of the bromide distribution ratios are greater than those for chlorides by an amount which is inexplicable on this hypothesis.

The work to be reported in this paper deals with (1) the development of an electrometric method for the determination of bromides

and chlorides in the presence of each other, (2) experiments on the distribution of bromide, chloride, and bicarbonate ions in blood to which sodium bromide had been added, and (3) the effect on the distribution ratios of varying the reaction of the blood.

Analytical Procedures

The reaction of the blood was determined in all instances by the colorimetric pH method of Hastings and Sendroy (2). In some cases determinations by the quinhydrone electrode were also made with Cullen's technique (3). The latter method gave values 0.09 pH lower than the colorimetric method but the variations from sample to sample were the same for both methods.

CO₂ analyses of serum and cells were made on the Van Slyke and Neill manometric blood gas apparatus (4).

H₂O determinations on serum and cells were obtained by drying a known volume of serum and a known weight of cells to constant weight at 100°.

Total halide, bromide, and chloride determinations were made by the method of electrometric titration described in a later section.

Experimental Technique

Throughout the experiments reported in this paper the blood used was obtained from dogs. The blood was collected and defibrinated under oil. In case it was to be equilibrated after the addition of sodium bromide, samples were transferred to 50 cc. centrifuge tubes with constricted necks. These were then attached by a 3 inch length of rubber tubing to tonometers of 250 cc. capacity and the desired gas mixture admitted from the gas manifold. After tipping the centrifuge tube until the blood passed to the large tonometer, equilibration was carried out in a large water thermostat, usually at 38°, for approximately 1 hour unless otherwise specified. At the end of this period, the blood was allowed to flow from the large tonometer to the attached centrifuge tube. The rubber tubing was doubled tightly across the top of the tube and wired in place. The centrifuge tube was then cut loose from the large tonometer and centrifuged. The serum and cells were separated and kept for analysis in small sampling vessels over mercury.

Electrometric Titration of Bromides and Chlorides in Serum and Cells

For the study of the problems under consideration in this paper a method for the determination of bromides and chlorides on small amounts of serum and cells was required. After reviewing the methods available for biological materials, the electrometric titration of the two halides in the presence of each other was decided upon. The principle of utilizing the differences in the solubility products of AgBr and AgCl to determine the end-point of the bromide titration is well recognized and has long been utilized by physical and analytical chemists. Since it has not been utilized much in biological work, however, the principle and technique involved will be given in some detail.

A silver electrode in equilibrium with silver ions will have a potential at 25° given by the following equation.

$$E_{\text{Ag}} = 0.059 \log_{10} \text{Ag}^+ + C \quad (1)$$

where

E_{Ag} = the potential in volts of the Ag-Ag⁺ half-cell

Ag⁺ = the activity of silver ions in the solution

C = a constant, numerically equal to the normal potential of the Ag-Ag⁺ electrode in a solution 1 N with respect to Ag⁺ ions

If a bromide solution is added to such a solution of silver ions there will be precipitation of silver bromide to a point such that the product of the silver and bromide ionic activities equals a constant known as the solubility product constant. This is expressed by the equation

$K_{\text{s.p.}} = (\text{Ag}^+) \times (\text{Br}^-)$ where

$K_{\text{s.p.}}$ = the solubility product constant

(Ag⁺) = the activity of silver ions in the solution

(Br⁻) = " " " bromide " " " "

Rearranging,

$$(\text{Ag}^+) = \frac{K_{\text{s.p.}}}{(\text{Br}^-)} \text{ and } E_{\text{Ag}} = 0.059 \log \frac{K_{\text{s.p.}}}{(\text{Br}^-)} + C$$

Thus it is seen that the potential of a silver electrode in equilibrium with solid AgBr is a function of the bromide ionic activity of the solution.

Instead of starting with a silver electrode in a solution of silver ions and adding bromide ions, one may start with a silver electrode coated with silver bromide in a solution of bromide ions and add silver ions. The relations are the same in both cases.

The theoretical potential at which the amount of silver added to the solution just equals the amount of bromide originally present may be calculated in the following manner.

Let Ag = total silver present whether as $AgBr$ or as Ag^+ ion, and let Br = total bromide present both as $AgBr$ and as Br^- ion, then

$$Ag = AgBr + Ag^+ \quad (2)$$

$$Br = AgBr + Br^- \quad (3)$$

By subtracting equation (3) from equation (2)

$$Ag - Br = (Ag^+) - (Br^-)$$

Therefore when $Ag = Br$, $(Ag^+) = (Br^-)$. But $(Ag^+) \times (Br^-) = K_{s.p.}$; hence at the end-point, $(Ag^+)^2 = K_{s.p.}$ or $(Ag^+) = \sqrt{K_{s.p.}}$.

Substituting in equation (1) one finds that

$$E_{Ag} = \frac{0.059}{2} \log K_{s.p.} + C$$

That this is also the point of inflection of the curve obtained by plotting total silver added against E.M.F. is shown in the following way.

$$Ag = AgBr + Ag^+$$

But

$$Ag^+ = 10^{\frac{E.M.F. - C}{0.059}}$$

$$\therefore Ag = AgBr + 10^{\frac{E.M.F. - C}{0.059}}$$

$$\text{But } AgBr = Br - Br^-$$

$$\therefore Ag = Br - \frac{K_{s.p.}}{Ag^+} + 10^{\frac{E.M.F. - C}{0.059}} \quad \text{or}$$

$$Ag = Br - \frac{K_{s.p.}}{10^{\frac{E.M.F. - C}{0.059}}} + 10^{\frac{E.M.F. - C}{0.059}}$$

Since the total bromide is a constant, one may differentiate Ag with respect to E_{Ag} , whence one finds

$$\frac{d\text{Ag}}{dE_{\text{Ag}}} = 0.43 \times 10^{\frac{E_{\text{Ag}} - C}{0.059}} - \frac{0.43 K_{\text{s.p.}}}{10^{\frac{E_{\text{Ag}} - C}{0.059}}}$$

Setting this equal to zero

$$E_{\text{Ag}} = \frac{0.059}{2} \log K_{\text{s.p.}} + C$$

which is identical with the potential previously calculated for the point at which the added silver equals the bromide present.

With the same reasoning but with the constants characteristic for silver chloride, the end-point for the silver chloride titration may be similarly calculated. Due to the fact that the solubility product constant AgBr is 4.4×10^{-13} and AgCl is 1.9×10^{-10} , one can titrate one in the presence of the other.

The solubility product of AgBr is 4.4×10^{-13} , of AgCl, 1.9×10^{-10} . The end-points for the titration of Br or Cl singly would occur at the E.M.F. values of -0.189 and -0.267 volts, respectively. The titration of bromide in the presence of chloride modifies the end-point of the former but not of the latter. This is readily seen from the following illustration. Suppose a certain concentration of bromide is being titrated in the presence of 0.1 M of chloride per liter. Since the solubility product of silver chloride is 1.9×10^{-10} , AgCl will be formed as soon as the silver ion concentration reaches a value of $\frac{1.9 \times 10^{-10}}{0.1} = 0.19 \times 10^{-10}$. This corresponds to an E.M.F. of -0.184 volts when measured against a saturated calomel cell. Obviously, therefore, the precipitation of AgCl will have begun before the theoretical end-point for AgBr (-0.189) is reached and slightly more AgNO₃ must be added to reach the first point of inflection than would be theoretically required by the bromide present.

It is possible to determine this amount quite accurately however.

To determine the magnitude of the correction to be applied to the bromide end-point, experiments were run first on salt solutions of varying bromide and chloride concentrations; secondly, on se-

rum and cells of varying bromide concentrations. The results of these experiments are shown in Fig. 1. The correction to be applied in cc. of AgNO_3 is plotted as ordinates against concentration of bromide present as abscissæ. Examples of chloride and bromide analyses on serum and cells by the method of electrometric titration and of the total halides by the Van Slyke and Whitehorn methods are given in Tables I and II.

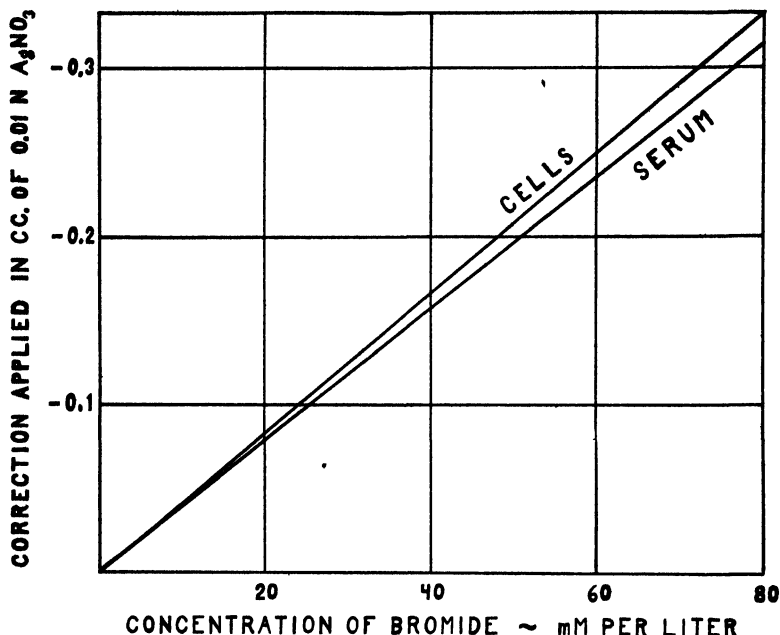


FIG. 1. Correction to be applied in the estimation of bromide (in the presence of chloride) by the electrometric titration of cell or serum filtrates.

Preparation of Filtrates for Titration

To 1 volume of serum (5 cc. are a convenient amount) are added 8.2 volumes of water. These are thoroughly mixed. While the flask is being agitated there are slowly added 0.4 volume of 10 per cent sodium tungstate, then 0.4 volume of $\frac{2}{3}$ N sulfuric acid. The mixture is filtered through a No. 50 Whatman filter paper.

The preparation of cell filtrates is similar except that different amounts of water and tungstic acid must be used. A weighed

amount of cells, about 5 gm., is transferred to a 1 liter Erlenmeyer flask. The approximate volume is calculated from the weight and specific gravity as the product of the weight times $\frac{1}{1.086}$. 15.6 volumes of water are added and the flask is agitated until hemolysis is complete. There are then added, in succession, 1.7 volumes of 10 per cent sodium tungstate and 1.7 volumes of $\frac{2}{3}$ N sulfuric

TABLE I

Comparison of Halide Determinations of Serum Made by Various Methods

Method	(Cl) initially	(Br) added	Total (Br) + (Cl)		(Br) found
			Calculated	Found	
	mm per l.	mm per l.	mm per l.	mm per l.	mm per l.
Electrometric titration.....	111 0	58 8	169.8	170.2	58.6
Van Slyke (condenser).....	110 7	58 8	169.5	172.0	
Alkali and acid ashing.....	111.8	58.8	170.6	171.7	
Whitehorn.....	113.4	58.8	172.4	172.6	

TABLE II

Comparison of Halide Determinations of Red Blood Cells Made by Various Methods

Method	(Cl) initially	(Br) added	(Br) + (Cl)		(Br) found
			Calculated	Found	
	mm per kg.	mm per kg.	mm per kg.	mm per kg.	mm per kg.
Electrometric titration.....	58.1	37.8	95 9	95.3	38.1
Van Slyke (condenser).....	50.6	30 0	80 6	83 8	
Alkali and acid ashing.....	56.1	37.8	93.9	94.5	
Whitehorn.....	59.4	37.8	97.2	96.9	

acid. The suspension is carefully filtered through a No. 50 Whatman filter paper. The resulting filtrate should be water-clear.

5 cc. of the serum filtrate or 15 cc. of the cell filtrate are measured into a 150 cc. beaker and diluted to 50 cc. with distilled water. The Ag-AgBr electrode is then immersed in the solution, connection is made with the calomel cell through an agar-KNO₃ bridge, and stirring is effected by a Cenco stirrer. The solution is titrated with 0.01 N AgNO₃, admitted in 0.2 cc. portions except near the

end-points when it is added drop by drop from a 10 cc. burette capable of being read to 0.01 cc. E.M.F. readings are made with a Leeds and Northrup type K potentiometer set-up. The titration results are plotted on cross-section paper with cc. of AgNO_3 as ordinates and E.M.F. as abscissæ. The points of inflection may be estimated to 0.02 cc. by geometrical analysis of the curves.

TABLE III

*Experiment on Distribution of Chlorides and Bromides in Blood
Equilibrated at 38° and Varying CO_2 Tensions*

In Samples 1 and 2, 50 cc. of a solution containing 129 mm NaCl + 25 mm NaHCO_3 per liter were added to 100 cc. of blood. In Samples 3 and 4, 50 cc. of a solution containing 129 mm NaBr + 25 mm NaHCO_3 per liter were added to 100 cc. of blood. November 11, 1927.

Sample No.	Specimen analyzed	pH*	H_2O		(Cl + Br)	(Br)	(Cl)
			gm. per cc.	gm. per gm.	mm per kg. H_2O	mm per kg. H_2O	mm per kg. H_2O
1	Serum	7.56	0.963		123.25		123.25
	Cells			0.698	79.4		79.4
2	Serum	7.35	0.965		120.0		120.0
	Cells			0.675	83.6		83.6
3	Serum	7.52	0.961		124.4	55.5	68.9
	Cells			0.672	79.2	36.9	42.3
4	Serum	7.36	0.968		123.3	52.4	70.9
	Cells			0.671	84.4	39.8	44.6
		pH*	$\frac{(\text{Cl} + \text{Br})_c}{(\text{Cl} + \text{Br})_s}$		$\frac{(\text{Br})_c}{(\text{Br})_s}$	$\frac{(\text{Cl})_c}{(\text{Cl})_s}$	
1		7.56	0.644			0.644	
2		7.35	0.696			0.696	
3		7.52	0.637		0.665	0.614	
4		7.36	0.684		0.760	0.629	

* Colorimetric, at 38°.

Results

Typical results found in the experiments on the distribution of bromide and chloride between serum and cells of oxygenated blood to which sodium bromide had been added and whose reaction had been altered by varying the CO_2 tension are given in Table III. Normal dog blood was divided into two 100 cc.

portions. To one were added 50 cc. of a solution containing 129 mm of NaCl and 25 mm of NaHCO_3 per liter; to the other, a solution containing 129 mm of NaBr and 25 mm of NaHCO_3 per liter. Such salt solutions are isotonic with blood serum and due to the presence of the NaHCO_3 maintain the HCO_3^- concentration at a normal figure. Each of these mixtures of blood and salt solution was equilibrated at 38° for 1 hour at CO_2 tensions of approximately 25 and 35 mm. of Hg. The serum and cells were then separated, transferred to tonometers over Hg, and the water, chloride, and bromide of each were determined by the methods previously described. All results are reported in terms of mm per kilo of water. In Table III the distribution ratios of bromide and chloride between serum and cells have been calculated. In the case of the blood plus NaCl, varying the pH from 7.56 to 7.35 caused a rise in the chloride distribution ratio; *i.e.*, chloride migrated from the serum into the cells. In the case of the blood with added NaBr, lowering the pH from 7.52 to 7.36 caused a rise in the distribution ratios of both bromide and chloride. It is significant, however, that the distribution ratio of bromide is in each case higher than that for chloride. That the difference is outside of the limit of analytical error is shown by the fact that in Sample 3, the bromide concentration in the serum would have had to be 4.6 mm per liter more; *i.e.*, 60.1 instead of 55.5 mm per liter to lead to a bromide distribution ratio equal to that for chloride. This is outside the limit of error of the analytical method. Significant also is the fact that all such experiments, of which we have twenty-five, have been consistent in giving higher bromide than chloride ratios. In view of the fact that the chloride ratios are lower and the bromide ratios higher than the chloride ratios in blood without bromide, it would seem that the conclusion that the bromide displaced chloride from the cells is inescapable.

In Table IV another type of experiment is illustrated. Blood was drawn, centrifuged, and the serum separated. Sodium bromide, sufficient to give a concentration of 50 mm per liter of blood, was dissolved in the serum. The serum was returned to the cells and the blood equilibrated at 38° and 40 mm. of CO_2 tension. Samples were taken for analysis at the end of 5 minutes and at the end of $1\frac{1}{2}$ hours. From the fact that the concentration of bromide is practically as high in the cells after 5 minutes of equilibration as

after 90 minutes it is evident that the exchange takes place with great rapidity. As in the experiments of the other type, the bromide distribution ratio was found to be higher than the chloride ratio.

In Table V, there are given the results of an experiment designed to determine the rate of passage of bromide from bromide-containing cells to bromide-free serum, and from bromide-containing se-

TABLE IV

Experiment on Distribution of Chlorides and Bromides in Blood Equilibrated at 37° and 40 mm. CO₂ tension

The blood of Sample 1 served as a control. To the serum of Sample 2 were added 50 mm of NaBr per liter of blood; this serum was then mixed with the corpuscles and equilibrated. March 5, 1928.

Sam- ple No.	Time of equilibra- tion	Specimen analyzed	pH*	H ₂ O		(Cl + Br) _c	(Br)	(Cl)	$\frac{(Cl + Br)_c}{(Cl + Br)_s}$	$\frac{(Br)_c}{(Br)_s}$	$\frac{(Cl)_c}{(Cl)_s}$
				gm. per cc.	gm. per gm.	mm per kg. H ₂ O	mm per kg. H ₂ O	mm per kg. H ₂ O			
1		Serum	7.47	0.938	0.919	113.2		113.2			
		Cells			0.651	76.9		76.9	0.679		0.679
2	5 min.	Serum	7.47	0.942	0.923	193.9	78.1	115.8			
		Cells			0.595	129.0	54.0	75.0	0.666	0.692	0.648
	92 min.	Serum	7.43	0.939	0.920	189.2	76.2	113.0			
		Cells			0.615	132.9	57.8	75.1	0.702	0.758	0.664

* Colorimetric, at 38°.

rum into bromide-free cells. The chloride distribution in a sample of normal blood was first studied. Another portion of the same blood was divided into two portions. To one was added sodium chloride in a concentration of 50 mm per liter of blood, to the other was added sodium bromide in a similar concentration. The samples were then equilibrated with CO₂ at a tension of 35 mm. of Hg at 38° for 45 minutes. The two samples were then analyzed as in the previous experiments. In this experiment the total halides of each sample were approximately the same. The serum containing

TABLE V

Experiment in Vitro on Rate of Diffusion of Bromides and Chlorides from Serum into Cells and from Cells into Serum

Sample 1 consisted of blood to which were added 50 mm of NaCl per liter. Sample 2 consisted of blood to which were added 50 mm of NaBr per liter. All equilibrations were carried out at 38° and with a CO₂ tension of 35 mm. of Hg. April 25, 1928.

Time of equilibration	Specimen analyzed	pH*	H ₂ O		Cl + Br	(Br)	(Cl)	$\frac{(Cl + Br)_c}{(Cl + Br)_s}$	$\frac{(Br)_c}{(Br)_s}$	$\frac{(Cl)_c}{(Cl)_s}$	Hemato-crit, per cent cells
			gm. per cc.	gm. per gm.	mm per kg. H ₂ O	mm per kg. H ₂ O	mm per kg. H ₂ O				
45 min.	Normal serum	7.60	0.939	0.922	121.2	121.2	121.2	0.671		0.671	45.8
	Cells			0.651	81.4	81.4	81.4				
	Serum of Sample 1	7.45	0.939	0.921	185.3	185.3	185.3	0.708		0.708	41.6
45 min.	Cells			0.616	131.1	131.1	131.1				
	Serum "	7.45	0.936	0.918	185.5	65.8	119.7	0.692	0.722	0.674	40.9
	Cells "			0.616	128.2	47.5	80.7				
Serum without bromide mixed with cells containing bromide											
8 min.	Serum	7.35	0.935	0.920	185.5	16.4	169.1	0.706	0.836	0.693	
1 hr.	Cells			0.631	130.9	13.7	117.2				
	Serum	7.35	0.935	0.918	186.0	18.3	167.7	0.719	0.760	0.715	42.3
	Cells			0.631	133.8	13.9	119.9				
Serum containing bromide mixed with cells without bromide											
8 min.	Serum	7.35	0.937	0.919	183.6	45.6	138.0	0.726	0.761	0.714	
1 hr.	Cells			0.644	133.2	34.7	98.5				
	Serum	7.35	0.938	0.918	182.5	46.4	136.1	0.735	0.768	0.724	40.0
4 hrs.	Cells			0.640	134.1	35.6	98.5				
	Serum	7.34	0.938	0.918	182.6	47.0	135.6	0.749	0.805	0.729	
5 hrs.	Cells			0.634	136.7	37.85	98.85				
	Serum	7.32	0.941	0.916	181.5	45.7	135.8	0.787	0.870	0.751	41.1
	Cells			0.634	142.8	39.73	102.07				

no bromide was then mixed with the cells containing bromide for 1 hour. Analyses were made after 8 minutes and 1 hour. It is seen that the bromide rapidly passed from the cells into the serum and that the bromide distribution ratios are always higher than those for chloride. In that portion of the experiment in which serum containing bromide was mixed with bromide-free cells there was a rapid passage of bromide from serum into cells with a tendency for it to accumulate in a higher concentration within the cells as time went on. This is shown by the rise in the distribution ratio of bromide from 0.76 to 0.87 after 5 hours equilibration.

TABLE VI

Experiment to Study Distribution of Br, and Cl between Serum and Cells in Oxygenated and Reduced Blood at Different pH

March 13, 1929.

Sample No.	Specimen analyzed	Initial pO_2	Final pH*	(Br)	(Cl)	(Br + Cl)	$\frac{(Br)_c}{(Br)_s}$	$\frac{(Cl)_c}{(Cl)_s}$	$\frac{(Br + Cl)_c}{(Br + Cl)_s}$
				mm per kg. H_2O	mm per kg. H_2O	mm per kg. H_2O			
1	Serum	0	7.47	60.1	69.8	129.9	0.779	0.700	0.736
	Cells			46.8	48.8	95.6			
2	Serum	0	7.26	57.1	68.7	122.9	0.890	0.775	0.846
	Cells			50.8	53.2	104.0			
3	Serum	140	7.50	64.6	69.5	134.1	0.695	0.603	0.648
	Cells			44.9	41.9	86.8			
4	Serum	140	7.26	60.0	64.9	124.9	0.785	0.735	0.759
	Cells			47.1	47.7	94.8			

* Colorimetric, at 38°.

Effect of Oxygenation-Reduction and pH Change on Bromide and Chloride Distribution

Two experiments have been performed to determine the effect of oxygenation and reduction of blood at different pH on the chloride and bromide distribution between serum and cells. Since both experiments gave similar results only one will be reported here. The results of this experiment, given in Table VI, may be summarized in the following conclusions. The bromide and chloride distribution ratios are lower in oxygenated blood than in reduced blood at the same pH by amounts consistent with those

predicted by Van Slyke, Wu, and McLean. As in the previous experiments increasing the pH of either oxygenated or reduced blood causes a decrease in the distribution ratios of both bromide and chloride comparable to that predicted.

From the foregoing experiments the following conclusions may be drawn.

1. When bromide is added to blood *in vitro* it distributes itself in such a way that the distribution ratio of bromide between cells and serum is about 10 per cent higher than the corresponding chloride ratio.

2. It seems to be freely diffusible from cells into serum or from serum into cells.

3. If the pH of the serum of bromide-containing blood is altered, the distribution ratio changes in a direction and in an amount corresponding to that predicted by the Donnan equilibrium; *i.e.*, a higher pH leads to a lower ratio.

4. If blood containing bromide is oxygenated and reduced, the bromide distribution ratio changes in a manner consistent with that expected of diffusible ions; *i.e.*, reduction of blood leads to a higher ratio.

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STUDIES OF BROMIDE DISTRIBUTION IN THE BLOOD

II. THE DISTRIBUTION OF BROMIDES AND CHLORIDES IN THE BLOOD OF DOGS FOLLOWING THE ORAL ADMINISTRATION OF SODIUM BROMIDE

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When sodium bromide was administered to dogs by mouth, analysis of the blood revealed a much higher bromide and a lower chloride concentration within the red blood cells than would have been expected on the basis of the *in vitro* experiments. This phenomenon was studied systematically and the following experiments illustrate the results obtained.

Effect of a Single Dose of Sodium Bromide

A large dog was given 40 gm. of sodium bromide by mouth in 750 cc. of H₂O and samples of blood were withdrawn at frequent intervals thereafter for 26 hours. Analysis of the blood revealed that the bromide concentration in the serum and cells reached a maximum concentration about 1½ hours after the sodium bromide was administered. The highest bromide distribution ratio occurred ½ hour after the bromide was given. There was apparently a redistribution of the bromide during the succeeding period with a gradual decrease in the bromide ratio toward normal.

Effect of Repeated Doses of Sodium Bromide

When a single dose of sodium bromide was given repeatedly over several days it was found that the highest bromide distribution ratio occurred on the morning following the administration of the first dose.¹ Although the blood bromide concentration con-

¹ A blood sample was secured each morning.

TABLE I

Examples of Bromide-Chloride Distribution in the Blood of Dogs Fed Sodium Bromide

Dog No.	$\frac{[(Cl) + (Br)]_c}{[(Cl) + (Br)]_s}$	$\frac{(Cl)_c}{(Cl)_s}$	$\frac{(Br)_c}{(Br)_s}$	$\frac{(Br)_c}{[(Br) + (Cl)]_c}$	$\frac{(Br)_s}{[(Br) + (Cl)]_s}$	Remarks
1	0.754	0.579	0.926	62.0	50.5	
2	0.745	0.597	0.927	55.9	45.0	
3	0.727	0.727				Control observation
	0.769	0.582	0.937	64.2	52.6	Maximum observed replacement of Cl by Br in cells
	0.758	0.553	2.052	36.9	13.6	Highest $\frac{(Br)_c}{(Br)_s}$ ratio observed
	0.729	0.752	0.702	43.6	45.3	Lowest $\frac{(Br)_c}{(Br)_s}$ ratio observed
4	0.752	0.752				Control observation
	0.653	0.413	0.892	68.4	50.1	Maximum observed replacement of Cl by Br in cells
	0.796	0.559	1.459	48.4	26.4	Highest $\frac{(Br)_c}{(Br)_s}$ ratio observed
	0.711	0.650	0.788	48.7	44.0	Lowest $\frac{(Br)_c}{(Br)_s}$ ratio observed
9	0.722	0.722				Control observation
	0.706	0.396	2.000	55.2	19.5	Highest $\frac{(Br)_c}{(Br)_s}$ ratio observed
	0.716	0.441	1.126	63.2	40.2	Lowest $\frac{(Br)_c}{(Br)_s}$ ratio observed also maximum replacement of Cl by Br in cells
10	0.735	0.735				Control observation
	0.878	0.585	1.104	70.3	55.4	Maximum observed replacement of Cl by Br in cells
	0.733	0.575	1.634	33.2	14.9	Highest $\frac{(Br)_c}{(Br)_s}$ ratio observed
	0.778	0.708	0.822	57.3	53.6	Lowest $\frac{(Br)_c}{(Br)_s}$ ratio observed

tinued to increase there was a gradual drop in the distribution ratio. The bromide ratio was almost always higher than the chloride ratio however.

Observations were made of the maximum replacement of chloride by bromide capable of being produced in the blood. Examples of such data are given in Table I. Replacement of as much as 70.3 per cent of the total halide by bromide was encountered but no relationship was found between the distribution ratio and the degree of depression of the central nervous system.

DISCUSSION

Bromide distribution ratios as high as 2.0 were sometimes observed and ratios between 1.0 and 2.0 occurred quite commonly. The degree of discrepancy between the observed ratios and those predicted on the basis of the Donnan equilibrium is emphasized if one remembers that the normal chloride ratio is approximately 0.7. The chloride ratios observed in the blood of bromide-fed dogs were commonly less than 0.7 and often were in the neighborhood of 0.5. This provides a clue to a possible explanation for these apparently anomalous distributions. It had been previously found by electrometric measurements that the distribution of hydrogen ions between serum and cells is such that the ratio $\frac{(\alpha_{H^+})_s}{(\alpha_{H^+})_c}$ approximately equals 0.5 at pH = 7.4. It should also be noted that this ratio is the only ratio thus far made of ionic activity distributions between serum and cells. Other ionic distributions which have been studied have been those of chloride and bicarbonate which have the approximate values $\frac{(Cl)_s}{(Cl)_c} = 0.7$ and $\frac{(HCO_3)_s}{(HCO_3)_c} = 0.8$. These have been based on determinations of total chloride and bicarbonate analyses made on serum and cells. If one can accept the hydrogen activity ratio of 0.5 as correct, one must assume that the value

$$\frac{(\alpha_{Cl})_s}{(\alpha_{Cl})_c} = \frac{(\alpha_{HCO_3})_s}{(\alpha_{HCO_3})_c} = 0.5$$

and

$$\frac{(\gamma_{Cl})_s [Cl]_s}{(\gamma_{Cl})_c [Cl]_c} = \frac{(\gamma_{HCO_3})_s [HCO_3]_s}{(\gamma_{HCO_3})_c [HCO_3]_c} = 0.5$$

whence

$$\frac{(\gamma_{\text{Cl}})_c}{(\gamma_{\text{Cl}})_s} = 0.71 \text{ and } \frac{(\gamma_{\text{HCO}_3})_c}{(\gamma_{\text{HCO}_3})_s} = 0.62$$

TABLE II

Rate of Diffusion of Bromides and Chlorides from Serum into Cells and from Cells into Serum

Dog 17, normal; Dog 16, bromide-fed. May 17, 1928.

Time of equilibration	Specimen analyzed	pH	(H ₂ O)	(H ₂ O)	(Cl + Br)	(Br)	(Cl)	$\frac{[(\text{Cl}) + (\text{Br})]_c}{[(\text{Cl}) + (\text{Br})]_s}$	$\frac{(\text{Br})_c}{(\text{Br})_s}$	$\frac{(\text{Cl})_c}{(\text{Cl})_s}$
			gm. per cc.	gm. per gm.	mm per kg. H ₂ O	mm per kg H ₂ O	mm per kg. H ₂ O			
	Serum	7.35	0.930	0.918	125.43		125.43	0.716		0.716
	Cells			0.635	89.8					
	Serum	7.31	0.937	0.921	132.1	92.6	39.5	0.742	0.738	0.754
	Cells			0.678	98.17	68.4	29.8			
Serum of normal dog (Dog 17) mixed with cells of bromide-fed dog (Dog 16)										
10 min.	Serum	7.27	0.932	0.920	125.4	34.2	91.2	0.758	1.445	0.500
	Cells			0.678	95.0	49.4	45.6			
2 hrs. 24 min.	Serum	7.21	0.931	0.915	126.5	34.45	92.06	0.796	1.09	0.685
	Cells			0.680	100.6	37.56	63.04			
Serum of bromide-fed dog (Dog 16) mixed with cells of normal dog (Dog 17)										
10 min.	Serum	7.27	0.937	0.920	128.1	54.25	73.85	0.736	0.844	0.655
	Cells			0.674	94.2	45.8	48.4			
2 hrs. 32 min.	Serum	7.21	0.935	0.918	126.0	54.4	71.6	0.766	0.822	0.722
	Cells			0.706	96.5	44.75	51.75			

A comparison of the ionic strength of serum and cells shows that in so far as the salt environment is concerned there is little difference between them, and the authors were left without a rational reason for the difference between the activity coefficient of chloride in serum and cells. Experiments have been quoted in this paper which show that in the presence of bromides, the distri-

bution of chlorides reaches a value of $\frac{(\text{Cl})_c}{(\text{Cl})_s} = 0.5$, which is the value found for $\frac{(\alpha_{\text{H}^+})_s}{(\alpha_{\text{H}^+})_c}$. The following hypothesis is presented to account for these facts. The assumption is made that even in normal blood a certain proportion of the chloride of cells is present in non-ionic form but that chloride can be displaced from this non-ionic form by bromide. The chloride ions distribute themselves in conformity with the distribution predicted by the Donnan law and have a distribution ratio equal to that of the hydrogen ions, namely 0.5.

This explanation, although not as yet proved, would account for the observed facts. Until measurements of chloride and bromide activities are made, however, it cannot be known whether or not the above hypothesis is correct.

An additional experiment may be cited which was designed to test the validity of the hypothesis just presented. (Although numerous attempts were made, bromide ratios greater than unity were not found in blood to which sodium bromide was added *in vitro*.) The purpose of this experiment was to determine the relative ease of diffusion of bromide from the cells of a bromide-fed dog into the serum of a normal dog, and conversely the ease of diffusion from the serum of a bromide-fed dog into the cells of a normal dog. The blood of a normal dog and a dog which had received 60 gm. of bromide over a period of 4 days was drawn and analyzed. Samples of each were centrifuged, the serum removed, and the bromide-containing serum mixed with the bromide-free cells and *vice versa*. These specimens of blood were then equilibrated under a CO_2 tension of 40 mm. of Hg at 38° for $2\frac{1}{2}$ hours. Samples were removed after 10 minutes and at the end of the experiment for analysis. The results are shown in Table II.

They show the following points of interest. The blood of the bromide-fed animal had a particularly high concentration of bromide in both the serum and cells, 70.1 per cent of the total halides of the serum, and 69.6 per cent of the halides of the cells being bromide. The bromide and chloride distribution in the blood as drawn were both practically normal and have the values 0.74 and 0.75 respectively. After 10 minutes of equilibration the bromide distribution in the blood composed of bromide-containing

cells and bromide-free serum was 1.45 and the corresponding chloride ratio was 0.5. After 2 hours and 24 minutes equilibration, the bromide distribution ratio had fallen to 1.09 and the chloride ratio had risen to 0.685. The bromide and chloride ratios of the blood composed of bromide-free cells and bromide-containing serum were respectively 0.84 and 0.66 after 10 minutes and 0.82 and 0.72 after 2 hours and 32 minutes equilibration. The latter ratios were approximately those encountered in blood to which bromide had been added *in vitro*, whereas the former ratios were much higher than those encountered in *in vitro* experiments and suggest a tendency on the part of cells from bromide-fed animals to retain their bromide.

THE INFLUENCE OF SODIUM TAUROCHOLATE AND COPPER SULFATE ON LIPASE

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The important results obtained by Rona and his collaborators (1-6) in the study of the lipases have prompted us to undertake some investigations along this line. We tried lipases of different origin; namely, water and glycerol extracts of fresh liver and pancreas that were obtained from the slaughter-house, a commercial preparation of liver lipase of the hog, serum lipase of rabbits, and we also studied an extract of adrenal glands. Furthermore, in our experiments we studied the influence of copper sulfate and sodium taurocholate on the lipases of these organs.

The experiments were carried out *in vitro* according to the method of Rona and Michaelis with the stalagmometer of Traube. We thus determined the rate of hydrolysis of tributyrin with the lipase alone and in the presence of copper sulfate or sodium taurocholate. In each experiment we used 50 cc. of a saturated solution of tributyrin, and, by means of 2 cc. of 0.3 M solution of a buffer consisting of primary and secondary sodium phosphates, we maintained the reaction during the experiment constant at pH 7.2. To different concentrations of the substratum and of the buffer solution we added weighed quantities of the preparation to be tested for lipase. The experiments were carried on at a temperature of 36.5°. Every 10 minutes a small volume of liquid was sucked into the stalagmometer and the number of drops counted.

Experiments with Extracts of Adrenal Glands

From the adrenal glands of cattle we prepared water and glycerol extracts and tested the hydrolysis of tributyrin, adding to the 50 cc. of tributyrin solution 0.25 to 0.5 cc. of the extracts that

corresponded to 0.1 to 0.15 gm. of fresh glands. After 2 hours at a temperature of 36.5° these extracts did not show any action on the tributyrin. These experiments were repeated several times and in no case could we discover the presence of lipase in the adrenal extracts. Using the same method of preparation, we obtained a liver extract that was rich in lipase and an adrenal extract entirely without lipolytic action. Furthermore, we determined whether or not adrenal extract exerts an inhibitory influence on the lipase. For this purpose we tested the hydrolysis of tributyrin with liver lipase alone and with a mixture of liver and adrenal extracts.

In both cases the lipolytic action of the liver extract remained the same. Therefore we conclude that the adrenal extract does not exert an inhibitory influence on the lipolytic power of liver

TABLE I
Action of Extracts of Liver and of Adrenal Glands

Temperature 36.5° ; pH 7.2.

Time after beginning experiment	50 cc. tributyrin, 2 " buffer, 1 " liver extract 1:4	50 cc. tributyrin, 2 " buffer, 1 " adrenal extract 1:4	50 cc. tributyrin, 2 " buffer, 1 " liver extract 1:4 1 " adrenal extract 1:4
min.	per cent	per cent	per cent
0	100	100	100
10	55	96.5	53.4
20	44.8	96.5	44.8
30	37.9	96.5	39.6

lipase. Our experiments demonstrate also the fact that the adrenal gland does not possess any lipolytic power. The results of these experiments are shown in Table I.

Influence of Sodium Taurocholate on Lipase of Liver and Pancreas

The influence of sodium taurocholate on hydrolysis of tributyrin is shown in Table II. For this experiment we prepared a suspension containing 10 mg. of hog liver lipase (commercial preparation) in 15 cc. of 10 per cent glycerol.

These experiments were repeated many times. In every case we found that sodium taurocholate in concentrations of 1:50,000, 1:100,000, and 1:500,000 considerably accelerated the hydrolysis of tributyrin by the lipases of liver and of pancreas.

Influence of Copper Sulfate on the Action of Lipase of Liver and Pancreas

Table III shows the influence of copper sulfate on the hydrolysis of tributyrin by liver lipase. In these experiments lipase sus-

TABLE II

Influence of Sodium Taurocholate on Liver Lipase

Temperature 36.5°; pH 7.2.

Time after beginning experiment	50 cc. tributyrin, 2 " phosphate buffer, 0.5 " lipase in suspension (a)	Na taurocholate (1:100,000)
		(a) plus 0.5 cc sodium taurocholate (1:1,000)
min.	per cent	per cent
0	100	100
10	81	60
20	63	52
30	56	41

TABLE III

Influence of Copper Sulfate on Liver Lipase

Temperature 36.5°; pH 7.2.

Time after beginning experiment	50 cc tributyrin, 2 " phosphate buffer, 0.5 " lipase in suspension (a)	CuSO ₄ · 5H ₂ O (1.100,000)	CuSO ₄ · 5H ₂ O (1.500,000)
		(a) plus 1 cc. CuSO ₄ · 5H ₂ O (1.2,000)	(a) plus 1 cc CuSO ₄ · 5H ₂ O (1.10,000)
min.	per cent	per cent	per cent
0	100	100	100
10	67	74	74
20	52	63	67
30	45	60	60
40	41	52	

pension prepared from commercial brands, as described above, was used.

In all of our experiments copper sulfate inhibited the action of liver lipase as well as that of pancreas lipase. The inhibitory action was manifest in the following concentrations of copper sulfate: 1:50,000, 1:100,000, and 1:500,000.

Influence of Copper Sulfate and Sodium Taurocholate on Pancreas Lipase

Table IV shows the influence of copper sulfate and sodium taurocholate on the activity of the pancreas lipase. The copper sulfate

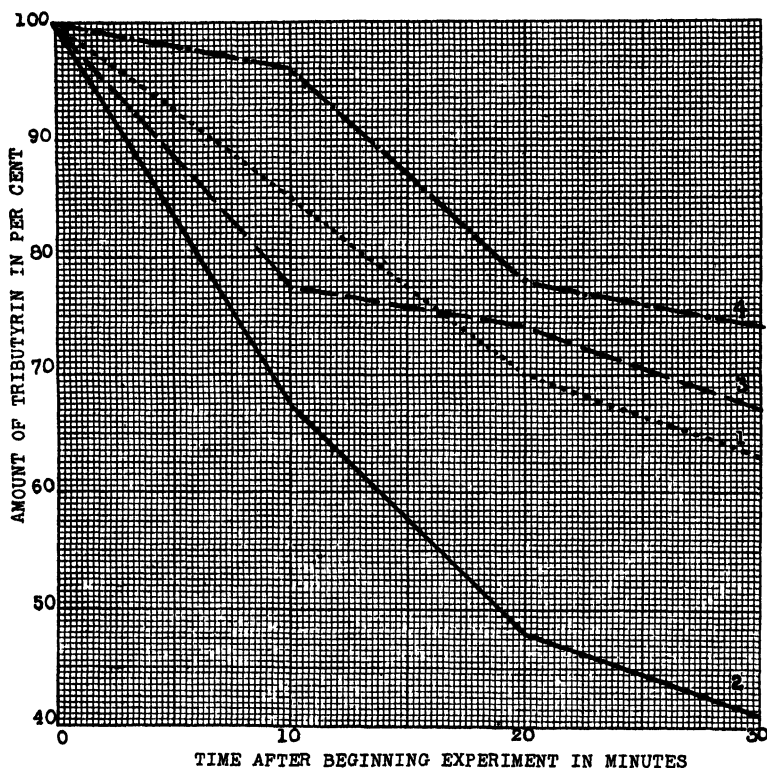


FIG. 1. The antagonistic action of copper sulfate and sodium taurocholate on hydrolysis of tributyrin by pancreatic lipase. Curve 1, control; Curve 2, with sodium taurocholate; Curve 3, with copper sulfate and sodium taurocholate; Curve 4, with copper sulfate. Temperature 36.5°; pH 7.2. Sodium taurocholate, 1:50,000; copper sulfate, 1:50,000.

considerably inhibited the work of pancreas lipase. On the contrary, the stimulating effect of Na taurocholate on the action of pancreas lipase is very pronounced.

Antagonism between Copper Sulfate and Sodium Taurocholate in Lipolysis

Further experiments were carried out on the antagonistic action of copper sulfate and sodium taurocholate. In these experiments

TABLE IV

Influence of Copper Sulfate and Sodium Taurocholate on Pancreas Lipase
Temperature 36.5°; pH 7.2.

Time after beginning experiment	50 cc. tributyrin, 2 " phosphate buffer, 1 " glycerol pancreas extract = 0.3 gm. fresh pancreas (a)	CuSO ₄ · 5H ₂ O (1:50,000)	50 cc. tributyrin, 2 " phosphate buffer, 0.5 " glycerol pancreas extract = 0.15 gm. fresh pancreas (b)	Sodium taurocholate (1:50,000)
		(a) plus 1 cc. CuSO ₄ · 5H ₂ O (1:1,000)		(b) plus 1 cc. sodium taurocholate (1:1,000)
min.	per cent	per cent	per cent	per cent
0	100	100	100	100
10	81	85	85	67
20	52	78	70	48
30	48	67	63	41

TABLE V

Influence of Sodium Taurocholate and Copper Sulfate on Serum Lipase
Temperature 36.5°; pH 7.2.

Time after beginning experiment	50 cc. tributyrin, 2 " phosphate buffer, 1 " rabbit serum (1:7) (a)	Sodium taurocholate (1:50,000)	50 cc. tributyrin, 2 " phosphate buffer, 1 " serum lipase (1 serum : 8H ₂ O) (b)	CuSO ₄ · 5H ₂ O (1:50,000)
		(a) plus 1 cc. sodium taurocholate (1:1,000)		(b) plus 1 cc. CuSO ₄ · 5H ₂ O (1:1,000)
min.	per cent	per cent	per cent	per cent
0	100	100	100	100
10	63	63	92	95
20	41	44	90	90
30	33	31	86	86

first sodium taurocholate and copper sulfate, and subsequently the lipase preparation, were added to the tributyrin solution. In Fig. 1 the results of one of these experiments are shown.

From these and other analogous experiments we conclude that the addition of sodium taurocholate to some extent protected the lipase from the inhibitory influence of copper sulfate.

Influence of Sodium Taurocholate and Copper Sulfate on Serum Lipase

In these experiments we used rabbit serum previously diluted 1:7 with water. Sodium taurocholate and copper sulfate in concentrations of 1:50,000, 1:100,000, and 1:500,000 were tested separately as to their influence on serum lipase. Table V shows the results of two of many similar experiments.

In accordance with this series of experiments it can be stated that copper sulfate and sodium taurocholate in the concentrations mentioned in the text have no effect on the serum lipase. The addition of glycerol in small quantities (0.2 to 50 cc.) to tributyrin, as was done in our experiment with the glycerol suspension of the commercial preparation of hog lipase, did not change the susceptibility of serum lipase toward either of the two substances.

DISCUSSION

A review of the literature (7-14) concerning the accelerating or inhibiting action of various substances on lipase indicates that our knowledge concerning the influence of various chemicals on lipase is in general inadequate and somewhat contradictory, although Rona and his collaborators (1-6) contributed much to our understanding of the action of some substances on lipase. As regards sodium taurocholate, many authors have noted its accelerating effect on lipase action. We found this statement to hold true only for liver and pancreas lipase, for we discovered that the serum lipase is not influenced by sodium taurocholate. We think it improbable, therefore, that, as Morse (15) believes, the accelerating effect of sodium taurocholate depends only upon its action on tributyrin.

There were no definite data that existed so far, concerning the effect of copper on lipolysis. We established the great toxicity of copper salts on liver and pancreas lipase, and their inhibitory effect even in very low concentrations on lipase action. Furthermore, we succeeded in diminishing this toxic effect of copper sulfate by the addition of sodium taurocholate. Our experiments show that, if we wish to explain the toxic action of copper on the organism, the influence of this metal on enzymes must be taken into consideration. In addition we were able to establish the specific

influence of copper sulfate on lipase of different origin. While it inhibited the action of liver and pancreas lipase, it had no effect on serum lipase.

In the literature dealing with lipase we found no data concerning the presence of this enzyme in the adrenal glands. Our experiments show that the adrenal glands of cattle are devoid of lipolytic action.

SUMMARY

1. Our experiments prove the absence of a lipolytic enzyme in the adrenal glands.

2. Sodium taurocholate acts differently on the lipase originating in various organs and on that lipase found in blood serum. It accelerates the activity of the pancreas and liver lipase and has no influence on the serum lipase; it accelerates the action of liver and pancreas lipase even in such low concentrations as 1:50,000 and 1:500,000.

3. Copper sulfate also acts differently on the lipase of certain organs and on that of the blood stream. It is toxic for liver and pancreas lipase and does not affect the serum lipase. This inhibiting action of copper sulfate is likewise evident in concentrations as low as 1:50,000 and 1:500,000.

4. The inhibitory action of copper sulfate on liver and pancreas lipase can be partly counteracted by the addition of sodium taurocholate.

5. From these findings it follows that the serum lipase is distinguished by its stability toward the compounds tested so far by us.

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THE DETERMINATION OF URIC ACID IN THE STUDY OF AVIAN NUTRITION*†

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The determination of uric acid plays an important rôle in the study of avian nutrition, and the comparatively small amount of work in the field of avian protein metabolism is partially due to the lack of a satisfactory method for ascertaining the amount of uric acid in the excreta. A method involving an operation upon the birds, such as used by Katayama (1924), Davis (1927), and Coulson and Hughes (1930), is questionable since metabolism must be affected by such an operation. The methods available for use directly on the normal excreta were those by Brown (1904), Bartlett (1910), Nitzescu (1918), Katayama (1924), and Woodman (1924).

Discrepancies in the results obtained by the latter method were found by Helphrey (1929) since the completion of the determinations by nitrogen determination, by titration with permanganate, and by the gravimetric method did not give concordant results. Inclusion of foreign material affected gravimetric results and the permanganate titration was affected by indistinct end-point and oxidizable material other than uric acid. Mitchell (1907), Stevens and May (1911), and Shimoda (1928) found that uric acid is decomposed by alkali. The Woodman method is also long and cumbersome. Heller, Morris, and Shirley (1930) had difficulty with Woodman's method, but their modification still retains much of Woodman's undesirable procedure. Both Brown's

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† We are grateful to Mr. Harold Gerritz for assistance on the analytical work on certain phases of this problem.

and Katayama's methods are time-consuming and filtration through coarse cloth in Brown's method could hardly be considered strictly quantitative and the titration end-point is difficult to determine.

As a result of the study of the various methods for the determination of uric acid in avian excreta, the following new method was developed. A weighed amount of the sample is placed in a 100 ml. volumetric flask. The amount of sample may conveniently be chosen so that the final precipitate of uric acid will be approximately 0.1 gm. although the method is equally accurate for wide variations in the weight of the final precipitate. 10 ml. of 0.15 N HCl are added and the flask held at boiling temperature for 3 to 4 minutes. The flask and contents are now cooled slightly and enough 0.25 N piperidine added to make the contents distinctly alkaline to phenolphthalein. After being heated for 45 minutes on a steam bath the solution is cooled, made to volume, and filtered. In cases where the clear solution can be decanted, it is sufficient to filter once on moderately fine textured paper. If the solution is cloudy or turbid, it may be advisable to filter first through a fluted filter and then through paper of finer texture. A 50 ml. aliquot of the filtrate is mixed with 16.5 gm. of ammonium chloride, held at 60-70° in a water bath for 20 minutes, and allowed to cool and crystallize for 24 hours. The solution is filtered on a small quantitative filter paper, the precipitate washed with 10 per cent ammonium sulfate solution and with distilled water, and finally washed back into the original beaker with a small quantity of hot 0.05 N HCl. If the solution is not distinctly acid to litmus, sufficient 20 per cent HCl is added to make it acid. The solution is then concentrated on a steam bath to 15 to 20 ml. and again allowed to crystallize for 24 hours. The uric acid is then filtered into a weighed Gooch crucible, dried at 100°, and weighed.

A second extraction with piperidine solution yielded no additional uric acid. No additional uric acid was obtained when the solutions were allowed to stand for as long as 6 days. An indication of the reliability of the method is shown by the close agreement between the results of duplicate determinations. On one series of thirty-four samples the average difference between duplicate determinations was 0.079 per cent. The maximum difference between any two duplicates was 0.22 per cent and the minimum

was zero. The duplicates were made on two separate portions weighed from each original sample and not on aliquots from the same solution, and covered a range of from 2.89 to 17.29 per cent of uric acid. On another series of twelve samples, results by another analyst showed a smaller difference of only 0.066 per cent

TABLE I
Purity of Uric Acid Precipitate As Shown by Nitrogen Determinations

Gravimetric	Kjeldahl	Difference
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1.56	1.57	0.01
2.83	2.77	0.06
4.20	3.97	0.23
5.34	5.26	0.08
5.36	5.12	0.24
5.60	5.50	0.10
5.61	5.48	0.13
8.47	8.19	0.28

TABLE II
Recovery of Known Amounts of Added Uric Acid

Sample No.	Weight of uric acid			Difference
	In original sample	Added	Recovered	
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
5	0.1836	0.0426	0.2256	0.0420
6	0.0788	0.0221	0.1013	0.0225
7	0.0630	0.0460	0.1083	0.0453
8	0.0630	0.1290	0.1925	0.1295
9	0.0847	0.0299	0.1152	0.0305
10	0.0847	0.0627	0.1480	0.0633

between duplicates. This method gave materially higher results than the Bartlett method.

The color and appearance of the precipitated uric acid indicated that it was practically free from foreign material. As a check on the purity a nitrogen determination was made on the precipitate remaining in the Gooch crucibles after the completion of the analysis. Typical results are shown in Table I.

A further check on the accuracy of the new method was made

by the analysis of samples containing known amounts of added uric acid. Results indicative of the amount of uric acid recovered are given in Table II.

This high recovery of the added uric acid indicates that there is no decomposition of the uric acid by the piperidine which is used as a solvent.

A modified method of handling the samples of fresh excreta is presented. The same samples were to be used for the determination of total nitrogen and ammonia nitrogen. To guard against the loss of ammonia nitrogen, acid was added at the poultry plant each day as soon as the sample was collected. Hydrochloric acid was used since this acid is also used in the analytical method for uric acid. When too much acid is used the sample will char during the last stages of drying and uric acid results will be affected. Bringing the sample to a pH of about 4.0 produced satisfactory results. This was done by adding 0.45 N HCl, with 1 ml. of this solution for each gm. of fresh excreta. The nutritional batteries used were described by St. John, Carver, *et al.* (1930). The pans are scraped carefully each day and the total weight of excreta determined. The sample is thoroughly mixed with the HCl and stored in a tared, covered, earthenware crock. At the end of the period the contents of the crock are thoroughly mixed and reweighed. A representative sample is placed in a 2 quart Mason jar and tightly covered. In the laboratory it is placed in a large evaporating dish and dried in a cabinet at 50° for 2 days, then in a drying oven at 105° for 10 hours. The percentage of dry matter and the total amount of dry matter for the period are calculated. The dry sample is ground in a Wiley mill to pass a 20-mesh sieve. Uric acid is unevenly distributed through fresh samples partly as crystals and partly as colloidal masses as noted by various authors (see Brown, 1904, and Gibbs, 1929). The above method of handling produces a very uniform sample.

Analysis showed that the same amount of uric acid was recovered irrespective of whether the determination was carried out on the fresh or on the dried sample. Uric acid was not lost on drying and neither does the drying in HCl give high results when this method is used. The same analytical procedure was used for moist and for dry samples.

SUMMARY

A method for the determination of uric acid in avian excrement is described. More accurate results have been obtained than with any other method used. The method is also more economical of time and reagents. A method of obtaining and preparing samples for analysis is described.

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STUDIES ON THE EFFECTS OF OVERDOSAGE OF VITAMIN D. II*

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(Received for publication, April 1, 1931)

In a previous communication from this laboratory (1) it was pointed out that extreme overdoses of vitamin D had a peculiar action on the mineral metabolism of white rats. Increasing overdoses of this vitamin, beyond a certain minimum, caused a progressive decrease in the ash of the long bones of the rats. These results were obtained after an overdose feeding period of only 2 weeks. Brown and Shohl (2) in similar experiments have confirmed and extended our findings. Their results show that excessive overdose produces very low calcium and phosphorus balances, in some cases negative, and gives low bone ash. Hypercalcification occurred at the epiphysis, which, together with the low bone ash, indicated demineralization of the shaft.

A number of investigators (3-12) have reported hypercalcemia, deposition of calcium in organs and tissues, and other pathological changes in rats receiving large overdoses of vitamin D for short periods.

In a study which we were conducting on the effect of low and high vitamin D overdose on the reproduction of white rats we had available a number of third and fourth generation animals whose progenitors had received 40 units and 2500 units of vitamin D supplementing the basal diet.¹ We, therefore, decided to use

* Read before the meeting of the American Chemical Society at Atlanta, Georgia, April 8-11, 1930.

¹ Bills' modification of the Steenbock formula:

Ground yellow maize.	76
Linseed oil meal.	16
Crude casein.	5

some of these animals in a study of the ash content of bones and of organs which we had found to be particularly susceptible to calcification, namely heart and kidneys.

We had available animals which could be classified in four groups as follows:

Group I—This group consisted of nine third and fourth generation young rats whose progenitors had received 40 units of vitamin D daily. This amount had been administered in the form of daily supplements of 0.4 gm. of dry irradiated yeast, 10 mg. of which, fed during a period of 10 days to rachitic rats receiving Steenbock Ration 2965, produced a continuous line of calcification. These animals were placed on the same basal ration¹ with the addition of 6000 units of vitamin D in the form of dry irradiated ergosterol crystals.² This ergosterol was dissolved in alcohol and mixed with the amount of food which the animal would consume each day, and the alcohol was allowed to evaporate. At the end of 3 weeks the animals were killed and autopsied, and the specified organs and bones were analyzed for ash content.

Group II—This group consisted of six adult second and third generation animals from the lot receiving 40 units of vitamin D daily. We determined the effect of this moderate overdosage of vitamin D on the ash content of the organs and bones under observation.

Group III—In our study on the effect of high overdosage of vitamin D on the reproduction of white rats, we obtained a number of third and fourth generation young animals whose progenitors had received 2500 units of vitamin D daily. These young animals had also from the weaning age received the same dosage. Only results of determinations on animals which had survived from 60 to 90 days were included in this study. Most of the animals died from the toxic effects of the overdosage. Seven animals are represented in the analyses given.

Group IV—In a previous investigation we had studied the effect

Ground alfalfa	2
Calcium carbonate.....	0.5
Iodized sodium chloride.....	0.5

To 3 parts of the above add 1 part of powdered whole milk.

² The irradiated ergosterol used in these experiments was activated by methods similar to those used in our previous experiments (1).

of a massive overdosage of vitamin D on the ash content of kidneys, hearts, and bones of normal young rats. These results have been included in Table I in order to compare the effect on the young of animals which have received a moderate overdosage of vitamin D through several generations. This group consists of eight normal young animals to each of which were administered 10,000 units of vitamin D daily for a period of 2 weeks.

TABLE I
Ash Analyses Based on Dry Weight

	Heart	Kidneys			Leg bones
		Right	Left	Average	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Group I	6.95	12.86	13.99	13.43	46.32
	4.51	9.25	9.66	9.46	47.71
	7.62	9.46	8.80	9.13	44.75
	6.93	7.25	7.45	7.35	44.70
	5.44	6.36	7.35	6.86	43.77
	6.38	12.04	10.75	11.40	46.72
	6.16	9.66	9.00	9.33	44.82
Averages of Groups I, II, III, and IV					
Group I.....	6.28			9.57	45.54
" II.....	2.54			3.10	62.21
" III.....	1.52			9.98	24.04
" IV.....	1.8			3.7	47.5

Analyses of bones and organs from all groups are given in Table I.

Results

Group I (Table I) shows the very marked effect of excessive overdosage on the ash content of the kidneys and hearts of these rats. During the 21 days the ash of the hearts increased from a normal of 1.5 to 2 per cent to values of from 4.5 to 7.6 per cent, while the kidneys increased from a normal of 2 to 3 per cent to values of from 6.4 to 14 per cent. The ash values of older animals (Group II) on the same basal ration and 40 units of vitamin D daily were from 2.2 to 3.0 per cent on the hearts, 2.75 to 3.75 per

cent on the kidneys, and 60 to 64 per cent on the bones. These results indicate that a long continued moderate overdosage of vitamin D in the form of irradiated yeast, whereby 40 units of vitamin D are administered daily from before weaning until old age, has no demonstrable effect on the mineral metabolism as determined by the ash of the hearts, kidneys, and bones. These animals, however, seem to be more susceptible to overdosages than normal animals. In previous work (Group IV) we found that the extreme overdosage of 6000 units of vitamin D per day for a 2 weeks period caused some demineralization of the bones, the ash dropping from 57 per cent to 47.5 per cent; but the ash of the hearts was normal, as was also the kidney ash except in one rat, where the kidney ash was 6.6 per cent. In the present series, Group I, the ash content of all kidneys and hearts was above normal, and the bone ash was invariably 15 to 20 per cent below normal.

In Group III, composed of animals from the reproduction experiment receiving the 2500 units of vitamin D daily, the young of the third and fourth generations showed striking effects from the overdosage. The parents at this time were healthy and normal so far as we were able to observe. In these young animals at about 45 to 60 days of age there was a pronounced flat-footedness and lameness in the hind legs resulting from what appeared to be a spontaneous fracture of the tibia. The animals were not excessively thin, and there was considerable fat present in the tissues. An excessive overdosage of vitamin D when given to normal animals causes an extreme emaciation and an almost complete absence of fat in the body. The presence of fat indicates an unusual effect of vitamin D overdosage. The abdomens of the animals were distended. The nose and feet in several cases showed lesions resembling pellagra.³ The hair was smooth and did not show the usual greasy, shaggy appearance of the coats of rats receiving a toxic overdosage of vitamin D. The heart ash of these Group III animals is about normal for their age (1.1 to 1.93 per cent), and in most cases the kidney ash is not abnormally high, al-

³ We have noted that animals receiving an overdosage of vitamin D drink about 50 per cent more water than animals on a normal diet. This observation is of interest in connection with the work of Cowgill, Rosenberg, and Rogoff (*Am. J. Physiol.*, **95**, 537 (1930)).

though in two instances ash values of 20.4 and 16 per cent were obtained. The most remarkable feature in this group is the extremely low bone ash, varying from 13.7 to 41 per cent with most of the values about 20 to 25 per cent. These determinations were made on animals from 60 to 90 days of age, at which age the normal bone ash is usually from 50 to 56 per cent. The bones which were so excessively low in ash presented an extreme picture of the condition described by Brown and Shohl. The head of the long bone showed hypercalcification while the shaft was so soft and decalcified that it could be easily bent with the fingers. This corresponds with the results of Brown and Shohl who stated that an overdosage of vitamin D caused a demineralization of the shaft of the long bones and a simultaneous deposition of calcium at the epiphysis.

SUMMARY

1. Moderate overdosages (40 units) of vitamin D daily have no effect on the mineral metabolism of white rats when continued through the third and fourth generations. No pathological calcification occurs and the ash of the bones is normal.

2. Animals receiving a moderate overdosage of vitamin D for a long period of time are more susceptible to a massive overdosage than normal animals.

3. A large overdosage (2500 units) of vitamin D just insufficient to produce toxic symptoms in the first and second generations, given for a long period of time, does produce striking pathological changes in the third and fourth generations. These changes are (a) decalcification of the bones, (b) severe calcification of the kidneys, and (c) certain pellagra-like symptoms, namely scabby conditions of the feet, nose, and forequarters.

We wish to acknowledge the valuable assistance of Mr. Wilfred Benson.

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SYNTHETIC NUCLEOSIDES*

III. THEOPHYLLINE-*d*-GLUCODESOSIDE

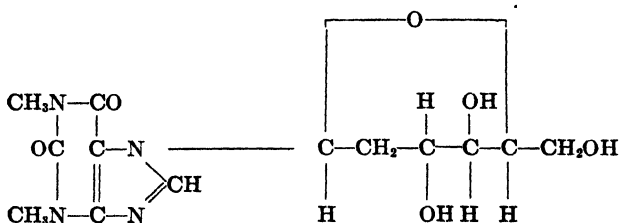
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(Received for publication, April 13, 1931)

The further knowledge of the details of the structure of thymus nucleic acid depends, in the first place, upon information regarding the details of the structure of the ribodesosenucleosides entering into its structure. However, the wider study of the structure of these substances is beset with many difficulties, as the naturally occurring substances are obtainable in small quantities only. Furthermore, the usual methods of determining the ring structure of glucosides were found ineffective when applied to nucleosides. It was thought, therefore, that the synthetic method might offer a better approach to the elucidation of those details in the structure of the nucleosides which as yet remain unknown.

Methods have been worked out for the preparation of nucleosides of normal sugars. Whether the same methods would lead to the synthesis of ribodesosenucleosides was not certain, particularly in view of the great instability of the desosides. The present communication contains a report on the synthesis of theophylline-*d*-glucodesoside. This was accomplished through the condensation of the silver salt of theophylline with 1-bromo-3,4,6-tribenzoyl-*d*-glucodesose which on saponification led to the desired nucleoside of the following structure.



* The first two publications in this series are: Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, **65**, 463, 469 (1925).

The details of the synthesis and of the physical properties of this substance are given in the experimental part. Work on the synthesis of ribodesosides is in progress.

EXPERIMENTAL

Tribenzoyl Theophylline-d-Glucodesoside—8.1 gm. of pure benzo-bromoglucodesose,¹ previously dried over phosphorus pentoxide for 1 hour under reduced pressure, were dissolved in 180 cc. of dry benzene. 9 gm. of the silver salt of theophylline, dried to constant weight at 130° under reduced pressure, were added and the mixture was boiled 10 minutes. The completion of the reaction was shown by the absence of a precipitate, when a drop of the reaction solution was shaken with a silver nitrate solution, acidified with nitric acid. The silver bromide was filtered and the almost colorless hot filtrate was treated with charcoal for 1 minute. Filtration now gave a colorless solution. On standing overnight at room temperature, 0.1 gm. of theophylline was deposited. The filtrate from this was poured into 2 liters of petroleum ether (b.p. 30–40°) and the whole thoroughly stirred. The fluffy white precipitate was allowed to settle and was washed twice by decantation with the precipitant. The flask was then put on the steam bath and acetone added. As the ether boiled away, more acetone was added, until complete solution occurred. 500 cc. of hot absolute alcohol were added, after concentration to a small volume. This was, in turn, concentrated until crystallization set in. 750 cc. of hot absolute alcohol were stirred in and the flask was stored overnight in the ice box. The solid mass of tiny hair-like silky needles was filtered, washed with petroleum ether, and air-dried several hours. The yield was 4.7 gm. or 73 per cent based on the benzo-bromoglucodesose.

This product contains no solvent of crystallization. It has no decisive melting point. It will shrink at about 140°, soften at about 150°, and the turbid melt will become clear between 170–193°, when effervescence also occurs. The behavior is probably due to gradual decomposition on heating. If the product is recrystallized from hot chloroform and excess cold ether, and the resulting needles are air-dried several hours, it will then show a

¹ Bergmann, M., Schotte, H., and Leschinsky, W., *Ber. chem. Ges.*, **56**, 1055 (1923).

melting point of 122°. This is fairly consistent. However, the crystals now have about 8 per cent solvent, and if this be driven off at 80° under reduced pressure, the crystals will then give the same ragged and unsatisfactory melting point as the original product. Recrystallization from boiling pyridine and excess cold ether also furnishes a product melting at about 122°. Vacuum drying of this again restores the original ragged melting point.

The product was recrystallized twice from chloroform and ether. The crystals were dried to constant weight at 80° under reduced pressure.

Rotation and Analysis

$$[\alpha]_D^{25} = \frac{+0.33^\circ \times 100}{1 \times 2.32} = +14.2^\circ \text{ (in } s\text{-tetrachloroethane)}$$

4.421 mg. substance: 10.355 mg. CO₂ and 1.945 mg. H₂O.

4.949 " " : 0.382 cc. N₂ (756 mm., 24°).

C₂₄H₃₀N₄O₈. Calculated. C 63.95, H 4.70, N 8.78

Found. " 63.87, " 4.92, " 8.82

Tribenzoyl theophylline-*d*-glucodesoside is insoluble in water, ethyl alcohol, ether, and petroleum ether. It is soluble in acetone, benzene, chloroform, and pyridine. It crystallizes in needles, which form in rosettes.

Theophylline-d-Glucodesoside—The usual methods of hydrolysis with ammonia fail to split off the benzoyl groups from tribenzoyl theophylline-*d*-glucodesoside. A more drastic method had to be resorted to, and a rather poor yield of the nucleoside was obtained.

3.5 gm. of tribenzoyl theophylline-*d*-glucodesoside were suspended in a filtered solution of 2.8 gm. of crystalline barium hydroxide in 75 cc. of anhydrous methyl alcohol. After 2 minutes boiling, complete solution had taken place. A strong ester odor indicated that the benzoyl groups had been split off to form methyl benzoate. As refluxing continued, a heavy precipitation gradually occurred. After 1 hour, the odor of methyl benzoate had disappeared. The flask was cooled under the tap, and the mixture of theophylline-*d*-glucodesoside and barium benzoate was filtered, washed with methyl alcohol, ether, and air-dried. The mixture amounted to 3.4 gm. The colorless methyl alcoholic filtrate was stored overnight in the ice box and yielded 0.2 gm. of theophylline-*d*-glucodesoside in small colorless cubes and rectangles.

The mixture of nucleoside and barium benzoate was triturated with 15 cc. of cold water to remove the major portion of the latter. The residue (1.5 gm.) was dissolved in hot water and the solution cooled to 0°.

Sulfuric acid was cautiously added to a faint excess. After filtering the mixture of barium sulfate and benzoic acid, ammonia was added to a slight excess to prevent any possible subsequent hydrolysis. The solution was now evaporated to dryness under reduced pressure in a bath maintained at 35–40°. The residue amounted to 0.9 gm. This was triturated with 2 N ammonium hydroxide, filtered, and washed with 2 N ammonium hydroxide, water, acetone, and ether. This procedure removed the ammonium sulfate and ammonium benzoate. The residue of pure nucleoside amounted to 0.3 gm.

The total yield was, therefore, 0.5 gm. or about 30 per cent.

The nucleoside was recrystallized from hot water and acetone, into tiny cubes and rectangles, which contained no water of crystallization. The cubes were actually rhombs with almost equal axes. It melted at 258° without decomposition. The presence of a slight amount of impurity markedly affected the melting point. A sample with 0.3 per cent ash melted at 203° with decomposition. Another slightly impure sample melted at 251° with decomposition but the micro analysis of the sample detected no ash, and the carbon, hydrogen, and nitrogen values were excellent.

Rotation and Analysis

$$[\alpha]_D^{25} = \frac{-0.28^\circ \times 100}{1 \times 1.04} = -26.9^\circ \text{ in water}$$

4.103 mg. substance: 7.237 mg. CO₂ and 1.930 mg. H₂O.

5.355 " " : 0.792 cc. N₂ (752 mm., 24°).

4.170 mg. substance: 7.298 mg. CO₂ and 2.070 mg. H₂O.

4.195 " " : 0.628 cc. N₂ (752 mm., 24°).

C ₁₃ H ₁₈ N ₄ O ₆ .	Calculated.	C 47.85,	H 5.52,	N 17.18
	Found.	" 48.09,	" 5.26,	" 16.82
		" 47.72,	" 5.52,	" 17.02

The nucleoside is only slightly soluble in cold water, but soluble in hot water and in hot pyridine. It is insoluble in methyl and

ethyl alcohols, ether, petroleum ether, acetone, benzene, and chloroform. It is tasteless. It is stable in boiling Fehling's solution. A 1 minute boiling with 2 N hydrochloric acid causes hydrolysis, as shown by subsequent reduction of Fehling's solution. The nucleoside is apparently stable toward boiling with 0.05 N hydrochloric acid for 1 minute.

COLORIMETRIC METHOD FOR QUANTITATIVE DETERMINATION OF IRON IN BLOOD IN THE FORM OF DISPERSED PRUSSIAN BLUE

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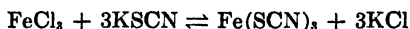
A simple rapid method for quantitative determination of iron in blood and in other organic bodies may lend itself to important investigations. Several methods of determination exist in the literature, but almost all of them involve the incineration of a given amount of blood, extraction of the iron from the ash, and then its estimation by titration or colorimetry. They seem to be too complicated to be adaptable. For this reason we are presenting a rapid colorimetric method for determining the iron content of the blood by digestion in an oxidizing mixture and subsequent comparison against a standard iron solution as Prussian blue in a dispersing medium of gum ghatti (1).

Gum ghatti was introduced by Dr. Folin in his new method of blood sugar determination as a dispersing medium.

In the digestion of the blood we were using a mixture of concentrated sulfuric and nitric acids, adding in case of need a small amount of powdered potassium chlorate. Later we found that Wong (2) used concentrated sulfuric acid and saturated potassium chlorate solution, while Kennedy (3) used concentrated sulfuric and perchloric acids. We adopted Wong's method of digestion.

Wong determines colorimetrically the iron content of the digested fluid in the form of ferric thiocyanate against a standard iron solution. He does not treat his standard solution in the same way as his sample, therefore his standard develops somewhat stronger color due to the fact that the sample contains additional potassium salt from added potassium chlorate which causes

reversion of the reaction toward the left according to the well known equilibrium,



Moreover, the color of thiocyanate progressively fades, while Prussian blue is almost permanent and offers a sharper and more delicate comparison.

The solutions required are (1) standard ferric sulfate solution, (2) saturated solution of iron-free potassium chlorate (about 8 per cent), (3) 6 per cent solution of iron-free potassium sulfate, (4) 1 per cent potassium ferrocyanide in gum ghatti solution.

The standard ferric sulfate solution is prepared as follows: Place in a standard liter flask 3.5575 gm. of anhydrous ferric sulfate (or 5 gm. of 9 M hydrate); dissolve it in about 100 cc. of distilled water with the aid of 10 cc. of iron-free concentrated sulfuric acid, cool, and bring to volume. The solution will contain 1 mg. of iron per cc. From this stock solution remove with a pipette 10 cc. to a 100 cc. standard flask and dilute to volume. This solution contains 0.1 mg. of iron per cc.

Anhydrous ferric sulfate being somewhat hygroscopic, the use of an old sample of the salt should be avoided, or else the concentration of the prepared standard solution should be determined.

The preparation of second and third solutions needs no explanation.

The gum ghatti solution is prepared according to Folin's method. (10 gm. of gum ghatti are suspended in a hollow copper gauze in 500 cc. of water in a cylinder or beaker and allowed to diffuse over a 24 hour period.) Subsequent oxidation with permanganate solution is omitted.

Place 5 gm. of potassium ferrocyanide in a 500 cc. standard flask, dissolve it in as little water as possible, and make to volume with gum ghatti solution which has been previously filtered through glass wool. Keep the solution in a brown bottle in the dark and occasionally bubble illuminating gas through it to prevent oxidation.

Procedure—Transfer 4 cc. of distilled water to a clean test-tube, add 1 cc. of blood, draining the pipette very slowly thus leaving the walls clean. Mix thoroughly. If the pipette is standardized with

a constant column of water at the tip, allow the same column of blood to remain. Remove 1 cc. of the diluted blood, corresponding to 0.2 cc. of the original blood, to a large Pyrex tube; or, if desired, 0.2 cc. of the original blood may be used.

Add to the blood 1 cc. of iron-free concentrated sulfuric acid. Insert a small Pyrex funnel and slant the tube at an angle of about 60°. Heat carefully until the water is driven off and then boil vigorously for 3½ minutes with a micro burner. This should be done either under the hood or under a larger inverted funnel connected to a suction tube. Do not use a bead unless it is of quartz, as ordinary glass beads leave considerable turbidity which is a serious detriment to analysis. Allow to cool for a few seconds, and add drop by drop through the funnel along the sides of the tube 1 cc. of saturated potassium chlorate solution. Boil for 3 minutes, again cool, add 0.3 cc. of chlorate solution, and boil for 2 minutes. Cool, wash the sides of the funnel into the tube to about 10 cc. volume.

Transfer to another Pyrex tube 1 cc. of the standard ferric sulfate solution containing 0.1 mg. of iron, add 1 cc. of concentrated sulfuric acid and 1.3 cc. of 6 per cent potassium sulfate solution (equivalent to the potassium chlorate used in the unknown), and dilute to about 10 cc. Add to each tube 1 cc. of the potassium ferrocyanide-gum ghatti solution, mix well, allow them to stand for 5 minutes, and dilute to the 25 cc. mark. Allow the solutions to stand for 15 minutes, and make the colorimetric comparison.

Solutions must be clear or error will result. If the tubes containing dispersed Prussian blue are heated in a boiling water bath for 5 to 6 minutes the color grows deeper, but the recovery of added iron becomes less efficient.

Calculation is according to the usual method.

$$\frac{\text{Reading of standard} \times \text{mg. Fe in standard} \times 100}{\text{Reading of unknown} \times \text{volume of blood used}} = \frac{20 \times 0.1 \times 100}{R \times 0.2} =$$

$$\frac{20 \times 50}{R} \quad \text{mg. iron per 100 cc. blood}$$

R is the reading of the unknown.

In the hope of diminishing the experimental error which might result from the use of small quantities of blood the amount of iron

in 1 cc. of whole blood was determined. To this were added 2 cc. of concentrated sulfuric acid and the mixture boiled for 4 minutes; the mixture was cooled, 1 cc. of saturated potassium chlorate solution was added through the funnel, and again boiled for 4 minutes; addition of 1 cc. of potassium chlorate was repeated three times more, with boiling for $3\frac{1}{2}$ minutes after each addition.

When doubtful as to complete oxidation of the mixture add 0.3 cc. more and boil for 2 minutes. Wash any possible adhering chlorate salt on the walls of the funnel with 1 cc. of water, by adding drop by drop from a pipette, and boil for 1 minute to decompose any trace of chlorate.

The standard against this sample contained 0.5 mg. of iron which was treated with 2 cc. of concentrated sulfuric acid and 4 cc. (or 4.3 as the case may be) of 6 per cent potassium sulfate (equivalent to chlorate).

The contents of each tube were diluted to about 15 cc., treated with 2 cc. of the potassium ferrocyanide-gum ghatti solution, and diluted to the 50 cc. mark.

The following tabulated data are representative of the results obtained from various determinations and indicate that there is no advantage in working with larger quantities of blood.

1	cc. whole blood (oxalated)	gave 47 2 mg. Fe per 100 cc. blood.
0.2	" " " " "	47.6 " " " 100 " "
1	" diluted blood (equivalent to 0.2 cc. whole blood)	gave 47 6 mg. Fe per 100 cc. blood.
1	cc. whole blood (oxalated)	gave 55 0 mg. Fe per 100 cc. blood.
0.2	" " " " "	55.0 " " " 100 " "
1	" diluted blood (oxalated) (equivalent to 0.2 cc. whole blood)	gave 55 5 mg. Fe per 100 cc. blood.

In a large number of experiments different samples of oxalated blood showed about 50 mg. of iron per 100 cc. of blood, in a few cases as high as 55 and 56 mg. Oxalated blood always gives lower values because of additional potassium or sodium salts and dilution. Difficulties arise from unsatisfactory matching of the colors due to turbidity from soft glass, metallic contamination from the cups (preventable by pipette transference), excessive oxalation of the blood, etc.

Determinations for the recovery of known amounts of added iron were also made. Table I shows the results obtained.

TABLE I
Recovery of Known Amounts of Added Iron

Experiment No.	Iron added to 0.2 cc. blood	Total amount of iron	Amount of iron found	Error
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1	0.01	0.11	0.110	0.0
2	0.01	0.11	0.110	0.0
3	0.01	0.11	0.111	0.9
4	0.02	0.12	0.120	0.0
5	0.02	0.12	0.121	0.8
6	0.02	0.12	0.121	0.8
7	0.03	0.13	0.132	1.5
8	0.03	0.13	0.131	0.8
9	0.03	0.13	0.130	0.0
10	0.04	0.14	0.140	0.0
11	0.04	0.14	0.140	0.0
12	0.05	0.15	0.148	1.3

SUMMARY

A new method is presented for the determination of the iron content of blood in the form of Prussian blue in a dispersing medium of gum ghatti solution.

The essential steps are the digestion of 0.2 cc. of blood with 1 cc. of concentrated sulfuric acid, boiling it for 3½ minutes, then addition, in two intervals, of 1 cc. and 0.3 cc. of a saturated solution of potassium chlorate boiling respectively 3 and 2 minutes; the conversion of the iron into Prussian blue by the addition of 1 per cent of potassium ferrocyanide in gum ghatti solution; finally the colorimetric comparison against a standard solution of ferric sulfate containing 0.1 mg. of Fe and 1.3 cc. of 6 per cent potassium sulfate.

Further study of the method and its application is being continued.

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THE CONJUGATION OF BENZOIC ACID IN MAN*

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The extensive investigations on the conjugation of benzoic acid, since Wöhler's discovery in 1824 of the synthesis of hippuric acid by the animal, have failed to furnish a comprehensive and definite concept of the metabolic and chemical changes taking place when benzoic acid is introduced into the organism. No attempt has ever been made to explain why man eliminates benzoic acid almost completely as hippuric acid while the dog excretes it mainly as glycuronic acid monobenzoate. Indeed many investigators have entirely ignored the fact that benzoic acid can be and is combined with glycuronic acid, and have recorded their determinations of total combined benzoic acid as hippuric acid. The seriousness of this error can be readily appreciated when one recalls that in the dog, glycuronic acid monobenzoate constitutes roughly three-quarters of the total combined benzoic acid, and that even in man varying amounts of benzoic acid combined with glycuronic acid may be excreted. The lack of a specific and reliable quantitative method until recently for the determination of hippuric acid has been another serious source of error.

The conjugation of benzoic acid constitutes a problem which deserves careful study because it offers a promising approach to the investigation of intermediary metabolism. The well known theory of β -oxidation of fatty acids was developed from the study of the fate of benzoic acid and its homologs in the body. It is highly probable that further work on the behavior of aromatic compounds, such as benzoic acid, which can elicit a physiological

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response without themselves becoming oxidized and completely destroyed, may furnish further significant facts that bear on the problem of the intermediary metabolism of both carbohydrates and protein. With this object in mind the present research was undertaken. It was realized however that before much progress could be made, it was essential to know more about the basic factors that influence the absorption, conjugation, and elimination of benzoic acid in both man and animals. Since the human and the dog differ most in their handling of benzoic acid, the work will be limited for the most part to these two species, and the present report deals exclusively with the problem of benzoic acid in man.

HISTORICAL

Normal Excretion of Hippuric Acid

The literature on benzoic acid is too extensive to allow a complete bibliography. Many papers, furthermore, are concerned with the fate of benzoic acid in the lower animals and have little bearing on the problem of the formation of hippuric acid in the human.

The earliest work was confined to the qualitative determination of hippuric acid following the ingestion of various substances. It was found that besides benzoic acid, other compounds, notably, benzaldehyde (1), cinnamic acid (2), toluene (3), and quinic acid (4), gave rise to an increased output of hippuric acid. Lücke (5) observed that the excretion of hippuric acid was also elevated after the ingestion of vegetables and fruits, especially cranberries. Blatherwick and Long (6) have found that a man may produce as much as 10 gm. of hippuric acid after eating 450 gm. of prunes and 4.7 gm. after consuming 305 gm. of cranberries. It is commonly accepted that quinic acid is the substance present in fruit which is responsible for the increased excretion of hippuric acid. The amount of preformed benzoic acid in fruits is too small to account for all of the hippuric acid. Blatherwick and Long found only 0.147 and 0.096 per cent respectively in prunes and cranberries. The benzoic acid from this source constitutes therefore only 6 per cent of the total amount excreted after eating prunes. Recently Kohman and Sanborn (7) have stated that they have isolated quinic acid from prunes and cranberries.

Salkowski (8) and Baumann (9) pointed out the possibility of benzoic acid originating from the bacterial decomposition of protein. The small amounts of hippuric acid that are excreted during fasting or on a diet restricted as to vegetables and fruit are undoubtedly due to the abnormal metabolism of amino acid containing the benzene nucleus, but this problem has never been intensively studied.

Toxicity of Benzoic Acid

The toxicity of benzoic acid is even now a mooted question. Wiley (10) stated emphatically that sodium benzoate even in small doses (0.9 to 2.5 gm.) frequently caused toxic symptoms especially nausea and vomiting, and concluded that benzoic acid added to food was injurious to health. The Referee Board of Consulting Experts composed of Chittenden, Long, and Herter (11), on the other hand, could find no deleterious effects from moderate doses (up to 4.0 gm. daily). The results reported by others are equally as contradictory. Meissner and Shepard (12) found that in certain individuals 5.7 gm. of sodium benzoate produced marked gastrointestinal disturbance, whereas Lewinski (13) and more recently Bignami (14) found individuals who could tolerate 25 to 40 gm. with no or only minor symptoms. Sodium benzoate has even been given therapeutically in doses as high as 12 gm. daily without ill effects (15).

The toxic symptoms appear to be of two kinds: first, those arising from the local irritation of the gastrointestinal mucosa. The common symptoms are nausea, vomiting, diarrhea, which is sometimes even bloody, and anorexia. The second type of disturbance arises presumably from the action of benzoic acid on the central nervous system. In man this is manifested as headache, tinnitus, vertigo, giddiness, and reflex vomiting. The more severe symptoms such as convulsions and respiratory failure have been observed only in animals

Source of Glycine

Many of the recent papers on benzoic acid have been devoted to a study of the synthesis of glycine, and naturally most of these investigations have been carried out on lower animals. A good review of the literature has been given by Griffith and Lewis (16)

and by Csonka (17). The more important facts at least tentatively established are: (1) The glycine employed for the synthesis of hippuric acid is partially synthesized, and partially obtained from the food. (2) Addition of glycine or of a protein rich in glycine accelerates the rate of hippuric acid excretion in the rabbit (16), increases the output of hippuric acid in the pig (17, 18), and to a less degree in the dog (19). Kingsbury (20) reported that he encountered two healthy individuals with subnormal output of hippuric acid following the ingestion of sodium benzoate, whose excretion became normal when glycine was added to the diet. (3) Glycocholic acid probably is not a precursor of glycine, and even it were, the question of the original source of glycine for glycocholic acid would not be answered. (4) The effect of benzoic acid on nitrogen metabolism is still incompletely understood. Much of the experimental evidence is of doubtful value because the hippuric acid is calculated from the total combined benzoic acid and no account is taken of the presence of glycuronic acid monobenzoate. Several important studies have been carried out on man. Lewis (21) found that after taking sodium benzoate a decrease in the excretion of urea and ammonia resulted. Shiple and Sherwin (22) made similar observations and further noted that the benzoic acid did not cause an increase in the total nitrogen output. Lewis and Karr (23) recorded a definite decrease in the excretion of uric acid which made them suspect that this substance might be the precursor of glycine. Swanson (24), however, found a concomitant increase in the blood uric acid but no increase in the urea. He concluded that glycine is probably synthesized from the constituents which are normally converted to urea. Bignami (14) from his experiments of feeding excessively large doses of sodium benzoate concluded that the body had 13 gm. of glycine and 4.0 gm. of glycuronic acid available for conjugation.

Site of Synthesis of Hippuric Acid

Since the classical work of Bunge and Schmiedeberg (25), the kidney has been considered the organ in which the conjugation of benzoic acid with glycine takes place. Snapper, Grunbaum, and Neuberg (26) have repeated these early experiments of perfusing the kidney of dogs with benzoic acid and glycine and have corrobo-

rated the older findings. They also found a similar synthesis taking place in the kidney of other species, and they made the remarkable and important observation that even the human kidney when perfused with glycine and benzoic acid synthesizes hippuric acid. Whether the kidney is the only organ which possesses this power is questioned by some authors. Kingsbury and Bell (27) recorded actual isolation of hippuric acid in the blood of double nephrectomized dogs, and Friedmann and Tachau (28) isolated hippuric acid after perfusing the liver of a rabbit with blood containing benzoic acid but no glycine. These authors could find no glycine in the liver, and they arrived at the significant conclusion that the glycine is synthesized in the liver in response to benzoic acid.

Attempts to use the conjugation of benzoic acid with glycine as a clinical test have not met with much success. The literature has been carefully reviewed by Bryan (29). The most important finding is the delayed excretion of hippuric acid in the type of nephritis which is characterized by a retention of nitrogenous substances, but there is no indication that the actual synthesis of hippuric acid is impaired. In fact Snapper and Grunbaum (30) found an increased concentration of hippuric acid in the blood stream after feeding benzoic acid to patients with contracted kidneys. Bryan believes that the excretion of free benzoic acid is an indication of liver impairment and accounts for it as an inability of the liver to mobilize glycine. Unfortunately no cognizance was taken of the possible presence of glycuronic acid monobenzoate which is readily hydrolyzed and can thus give rise to much free benzoic acid.

Excretion of Glycuronic Acid Monobenzoate

Senator (15) in 1879 noted a strong reducing substance in the urine of patients who were taking large doses of sodium benzoate. Later Kobert (31) considered the appearance of a reducing substance in the urine as a valuable sign of sodium benzoate intoxication. Lewinski (13) also noted that the urine of individuals who took large doses of sodium benzoate had reducing properties and was dextrorotatory. Magnus-Levy (32) in 1907 showed that the reducing substance was a compound of benzoic acid combined with glycuronic acid. Csonka (17) in 1924 studied the quanti-

tative relationship of benzoic acid conjugated with glycine and with glycuronic acid in the pig. In 1926, the writer (33) isolated the compound in pure crystalline form, studied its chemistry, and re-named it glycuronic acid monobenzoate.

It is generally considered that in man small doses of benzoic acid are quantitatively excreted as hippuric acid. Dakin (34) found only slight reduction in the urine of men who took 10 gm. of benzoic acid. Neuberg (35), however, found definite evidence that even when as small a dose as 5.0 gm. of benzoic acid is taken, a portion may be conjugated with glycuronic acid, and that the difference between the total combined benzoic acid and hippuric acid may be as great as 7 to 12 per cent. The writer (36) has found that when glycuronic acid monobenzoate is ingested in moderate amounts, none of it is excreted in the urine, but the output of hippuric acid is definitely increased. From this he concluded that the body can oxidize and utilize conjugated glycuronic acid, and that the amount of glycuronic acid appearing in the urine is not a measure of the amount synthesized, but rather of how much has escaped oxidation.

EXPERIMENTAL

Most of the data were obtained on the same subject, but the more important experiments were repeated on two other individuals. Sodium benzoate¹ was taken with 200 cc. of water in the morning immediately on rising, and no food was eaten until the experiment was completed. It was found, however, that a breakfast consisting of toast and coffee did not affect the results. The urine was collected hourly, and preserved with toluene.

Analytical Methods

Hippuric Acid—The method described by the author (19) was used. Because of the high concentrations of hippuric acid encountered in this work, 5 cc. samples were usually employed for analysis. After the hydrolysis of the hippuric acid with hydrochloric acid, and subsequent evaporation to dryness, the residue

¹ Benzoic acid was always ingested as sodium benzoate and never as the free acid. In this paper, however, all values for benzoic acid are expressed as such and not as sodium benzoate, in order to maintain a uniform and convenient system for recording and comparing quantitative data.

was dissolved in hot water, treated with decolorizing charcoal, and the filtrate analyzed by the formol titration method directly without diluting to a definite volume as originally proposed. The method was again critically checked, and a typical result on a sample of human urine which contained no glycuronic acid monobenzoate nor free benzoic acid was as follows:

By the method of Kingsbury and Swanson (37)..... 2.20 gm.
 " " author's method..... 2.17 "

Glycuronic acid monobenzoate was determined by the Shaffer-Hartmann method as previously described, free benzoic acid by the

TABLE I
Excretion of Hippuric Acid after Eating Quinic Acid and Prunes

Time	Benzoic acid excreted as hippuric acid	Remarks
<i>hrs.</i>	<i>gm.</i>	
0-4	0.16	6.0 gm. quinic acid taken
4-8	0.48	
8-12	0.60	
12-22	1.18	
22-27	0.26	
0-3	0.15	250 gm. prunes taken
3-13	1.25	
13-17	0.43	
0-8	1.01	500 gm. prunes taken
8-16	1.95	
16-24	0.90	

method of Raiziss and Dubin (38). Uric acid was determined by the Morris and Macleod method (39) with use, however, of Benedict's reagent (40).

DISCUSSION

Quinic Acid as a Precursor of Benzoic Acid

A study of the rate of hippuric acid elimination following the ingestion of prunes gives indirect evidence that the source of benzoic acid is quinic acid. As will be seen later, the excretion of ingested benzoic acid is very rapid, whereas the benzoic acid de-

rived from quinic acid is eliminated very slowly (see Table I). When 6 gm. of quinic acid were taken *per os*, only 0.15 gm. of benzoic acid was excreted during the first 4 hours. After that the rate gradually increased but the elimination was not complete even after 24 hours. When prunes were eaten similar results were obtained. There was at first a pronounced delay in the excretion of hippuric acid, followed by a slow but gradual increase in the rate and a prolonged period of excretion. It is interesting that Meissner and Shepard in 1866 noted qualitatively this prolonged excretion of hippuric acid following the ingestion of quinic acid. The cause for this delay is not known. It is unlikely that it is due to the slow absorption of quinic acid from the intestinal tract. In one experiment the prunes exerted a violent laxative effect which resulted in an apparent complete elimination of the prune residue in 4 hours; nevertheless, the excretion of hippuric acid continued for over 14 hours. It appears probable that the elimination of hippuric acid represents the rate with which quinic acid is reduced in the body to benzoic acid. It should also be stated that quinic acid may not exist free in fruits but is perhaps combined as a glucoside.

Glycine, as an Important Factor in the Synthesis of Hippuric Acid

The absorption of sodium benzoate from the intestines appears to be very rapid, and probably is many times faster than the excretion of hippuric acid. Therefore, after the first 30 to 60 minutes following the ingestion of the drug, the rate of absorption need not be considered a major factor limiting the synthesis and excretion of hippuric acid. In animals such as the rabbit and the dog, the rate of hippuric acid elimination is essentially the same whether sodium benzoate is given *per os* or parenterally, and it seems reasonable to assume that similar results could be obtained on humans.

The fundamental factor governing and limiting the formation of hippuric acid is the rate of the synthesis of glycine by the body. It can readily be seen by inspecting Chart I that the body does not have a store of preformed glycine. Benzoic acid itself appears to be the stimulus for the synthesis of this amino acid; the greater the quantity of benzoic acid administered, the greater the amount of glycine produced until the maximum capacity for this synthesis

is reached. Thus, by the simple experiment of feeding sodium benzoate one can determine not only the rate at which glycine is formed, but also ascertain the maximum quantity of glycine which can be produced per unit of time. Again referring to Chart I, attention is called to the fact that no matter how large the amount of benzoic acid administered, the hourly excretion of hippuric acid rises to a maximum and then remains constant until all but a small portion is eliminated. That this fixation of the excretion of hippuric acid is entirely due to a limitation of the synthesis of

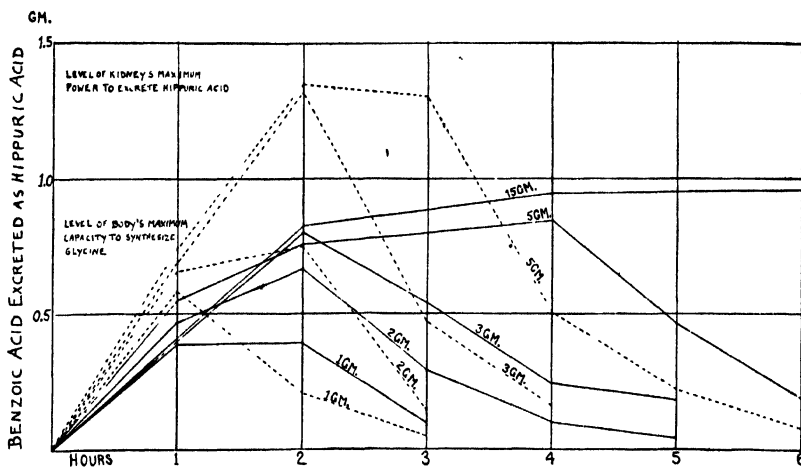


CHART I. The excretion of hippuric acid following the ingestion of benzoic acid in the fasting state, and when glycine is fed. The solid line indicates benzoic acid alone administered; the broken line, benzoic acid with glycine.

glycine can be conclusively demonstrated, for on the administration of glycine the hourly excretion of hippuric acid is promptly and decidedly increased (see Tables II to IV).

It has generally been accepted that the body can build up glycine, but the fact that the maximum rate of this synthesis can be definitely determined seems to have hitherto been overlooked. From the limited number of individuals studied, it was determined that the body can produce from 0.55 to 0.70 gm. of glycine per hour, the amount depending roughly on the surface area of the body. It is interesting to note here that Bignami (14) re-

ported that when he fed 20 to 40 gm. of benzoic acid to fasting men, only 21 gm. of benzoic acid appeared conjugated as hippuric

TABLE II

Excretion of Hippuric Acid after Ingestion of Benzoic Acid and of Hippuric Acid, with and without Glycine

Subject Q. Weight, 52 kilos; height, 164 cm.; surface area, 1.55 sq. m.

	Benzoic acid excreted as hippuric acid										
Benzoic acid taken....	1 gm.		2 gm.		3 gm.		5 gm.			Hippuric acid, 7.5 gm.*	
Time		With glycine†		With glycine†		With glycine†		‡	With glycine§		With glycine
hr.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	0.38	0.58	0.46	0.66	0.39	0.69	0.55	0.74	0.74	0.84	0.84
2	0.39	0.21	0.66	0.75	0.80	1.32	0.76	0.81	1.35	1.37	1.38
3	0.10	0.05	0.29	0.14	0.54	0.47	0.80	0.77	1.30	0.82	0.82
4			0.10		0.24	0.16	0.84	0.91	0.51	0.36	0.38
5			0.04		0.18		0.47		0.22	0.23	
6							0.19		0.08		

* Equivalent to 5.0 gm. of benzoic acid.

† 2.0 gm. of glycine.

‡ 0.5 gm. of theobromine taken at the beginning of the experiment.

§ 3.0 gm. of glycine.

TABLE III

Benzoic Acid Excreted as Hippuric Acid

Subject S. Weight, 58.0 kilos; height, 172 cm.; surface area, 1.70 sq. m.

Time	Urine volume	*	With glycine†	
			Urine volume	*
hr.	cc.	gm.	cc.	gm.
1	90	0.55	350	1.12
2	200	0.96	575	1.61
3	175	1.03	200	0.95
4	80	1.01	90	0.31
5	35	0.33	49	0.06
6	25	0.14		

* Took 5.0 gm. of benzoic acid.

† 3.0 gm. of glycine.

acid in 24 hours. This is equivalent to 13 gm. of glycine, a figure which agrees admirably with those found in the present investigation.

It is rather surprising that the potential capacity of the body to produce glycine should be so great. It is somewhat difficult to compare this synthetic power of man with that of the lower animals since very little data are available. From results reported by Griffith (41) it was calculated that the rate of the synthesis of glycine in the rabbit is about 0.025 gm. per hour per kilo of body weight. Similar calculations of Csonka's results (17) indicate that for the pig it is 0.015 gm. and from unpublished data it was determined that for the dog the rate is 0.0035 gm. per hour. On the basis of the data recorded in Table II, the rate of synthesis of glycine in man is approximately 0.009 gm. per hour per kilo of body weight. Thus, both the rabbit and the pig possess a rela-

TABLE IV
Benzoic Acid Excreted as Hippuric Acid

Subject W. Weight, 77.5 kilos; height, 188 cm.; surface area, 2.02 sq. m.

Time	Urine volume	*	With glycine†	
			Urine volume	*
hr.	cc.	gm.	cc.	gm.
1	120	0 85	130	1.22
2	99	1.11	130	1.84
3	72	0 63	130	0.80
4	53	0 61	130	0.30

* Took 5.0 gm. of benzoic acid.

† 3.0 gm. of glycine.

tively greater power to synthesize this amino acid than man, whereas the dog is distinctly limited in his ability to produce glycine.

Why the organism should possess such an efficient synthetic mechanism is rather difficult to understand. In the new born the necessity of the body to synthesize glycine is very evident, for this substance is needed in large quantities for growth, whereas milk, the sole food of the suckling, contains practically no glycine. It hardly seems probable however that this anabolic process has merely been carried over from early life. Glycine undoubtedly plays a more active physiological rôle than is generally appreciated. Obviously, a considerable quantity must be produced to form glycocholic acid, and it is suggestive that a

relationship exists between the dog's limited power to synthesize glycine and the absence or exceedingly low content of glycocholic acid in the bile of this animal. An interesting problem arises whether it is possible to change the relative proportions of glycocholic and taurocholic acid by either depriving the animal of glycine, which can be accomplished by feeding large quantities of benzoic acid, or by feeding excessively large amounts of glycine.

It seems quite probable that the prolonged feeding of benzoic acid can produce profound physiological disturbances which depend not primarily on the toxicity of benzoic acid, but rather on the diversion of glycine from normal metabolic channels. Thus, Griffith's results (42) on feeding sodium benzoate to growing rats kept on a diet low in glycine can perhaps be explained by the assumption that the capacity of the rat to synthesize glycine was sufficient to conjugate the benzoic acid but inadequate to supply the normal requirements. The beneficial results which he later obtained (43) by adding glycocholic acid to the diet are apt to depend more on the fact that the organism is relieved from the stress of furnishing glycine to produce this needed bile acid rather than that the latter compound acts as a precursor of glycine.

Another possible function of glycine must be considered. Brühl (44) from microscopic studies of the glomerular circulation of the frog has observed that a lack of glycine stops the circulation in the glomerulus. Similar observations were recorded earlier by Watzadse (45). In the light of these findings, an explanation may perhaps be found for the increased output of uric acid when glycine is fed as was observed by Lewis, Dunn, and Doisy (46), and recently corroborated by Christman and Mosier (47). Similarly, an answer may be found for the question why benzoic acid causes a sudden and marked drop in the excretion of uric acid.

Amino acids, of which glycine is no doubt the most available, seem to exert a direct stimulating effect on the kidney which manifests itself in an increase in the rate of excretion of uric acid. Amino acids can therefore almost be considered hormones to the kidney. Presumably, when the supply of exogenous amino acids is exhausted, glycine continues to be synthesized so that the excretion of uric acid proceeds normally. If, however, benzoic acid is administered, all of the glycine is diverted to be conjugated with the benzoic acid, thus producing an acute deficiency of glycine.

In the kidney this result produces a marked decrease in the excretion of uric acid, and as Swanson has shown, a piling up of the uric acid in the blood. If this hypothesis is correct, the drop in uric acid excretion should be prevented by supplying the organism with sufficient glycine before giving sodium benzoate. That this can actually be accomplished is seen by inspecting Table V. Instead of getting the usual decrease in uric acid excretion after giving sodium benzoate, an actual increase was effected by feeding as small a dose as 2 gm. of glycine 1 hour before giving 3 gm. of benzoic acid. The theory can be tested in another way. If uric acid is actually accumulating in the blood after the administration

TABLE V

Effect of Glycine in Preventing the Decrease of Uric Acid Excretion following the Administration of Benzoic Acid

Time	Urine volume	Benzoic acid excreted as hippuric acid	Uric acid output per hr.	Remarks
	cc.	gm.	mg.	
6 00- 8.00 a.m.	46		15.3	2.0 gm. glycine taken at 8.00 a.m.
8.00- 9.00	40		15.4	3.0 gm. benzoic acid taken at 9.00 a.m.
9.00-10.00	40	0.80	18.8	
10.00-11.00	50	0.96	13.0	

of sodium benzoate, then a subsequent excess dose of glycine should cause a markedly increased elimination of uric acid. The results recorded in Table VI indicate the correctness of this assumption. Following the ingestion of 6 gm. of benzoic acid the hourly excretion of uric acid dropped to one-third of the normal. After the subsequent administration of a relatively small amount of glycine, 5.0 gm., a portion of which was moreover rendered inactive by conjugation with benzoic acid, a striking increase in the elimination of uric acid resulted.

These considerations may throw some light on the perplexing observations of Harding, Allin, Eagles, and Van Wyck (48) who found that diets which contain sufficient excess fat to produce ketosis increased the concentration of uric acid in the blood while

carbohydrates following such a diet lowered the uric acid of the blood and caused an increased elimination in the urine. It is quite possible that these findings may be explained by the theory of glycine deficiency. If this is true, it further suggests that glycine may be synthesized from a carbohydrate derivative, and that when a deficiency of available carbohydrates occurs as indicated by ketosis, the synthesis of glycine is diminished or entirely stopped. Probably glycuronic acid is another factor which must be considered. This problem will be investigated and reported later.

TABLE VI

Stimulating Effect of Glycine on Excretion of Uric Acid Following Its Suppression by Benzoic Acid

Time	Urine volume	Benzoic acid excreted as hippuric acid	Uric acid output per hr.	Remarks
	cc.	gm.	mg.	
7.00- 8.00 a.m.	54	0.02	22.5	
8.00- 9.00	69	0.67	12.5	3.0 gm. benzoic acid taken at 8.00 a.m.
9.00-10.00	52	0.75	6.9	3.0 gm. benzoic acid taken at 9.00 a.m.
10.00-11.00	160	1.34	10.0	5.0 gm. glycine taken at 10.00 a.m.
11.00-12.00	202	1.34	44.7	
12.00- 1.00 p.m.	51	0.41	20.0	

Excretion of Hippuric Acid

The second most important factor in the elimination of benzoic acid from the body is the capacity of the kidneys to excrete hippuric acid. Obviously, from the foregoing discussion it was seen that the hippuric acid elimination is faster than the synthesis of glycine required for the formation of hippuric acid. With an adequate quantity of glycine supplied in the diet, however, the excretion of hippuric acid reaches a maximum rate, which represents the ultimate power of the kidney to eliminate this product. Again the quantitative results indicate that the maximum rate of excretion depends on the size of the individual, that is, on the surface area. The urine volume does not appear to be of any

significance. As can be seen from Table II, the excretion of hippuric acid is essentially the same whether the compound is ingested directly, or whether benzoic acid with an adequate amount of glycine is fed. Glycine, furthermore, when administered with hippuric acid exerts no influence on its rate of excretion. This is contrary to Griffith's (49) observation on the rabbit, for in these animals glycine definitely did increase the rate of the elimination of ingested hippuric acid, but according to this author, hippuric acid is partially hydrolyzed in the intestines. Such a hydrolysis is rather unlikely in man since the absorption of hippuric acid is rapid, and it was furthermore found that a closely related substance, phenylaceturic acid, is excreted unchanged, whereas if it were hydrolyzed the phenylacetic acid should appear combined with glutamine (50).

The maximum output of benzoic acid as hippuric acid when the body has sufficient glycine varied in three individuals from 1.40 to 1.82 gm. per hour. Especially striking was the constancy of the maximum hourly output of hippuric acid in Subject Q in a series of experiments of rather widely varying conditions. Contrary to the findings of Lewis (21), Kingsbury and Swanson (37), and Bryan (29), the elimination of benzoic acid was rarely over 75 to 80 per cent complete in 6 hours. In agreement with Snapper (51) it was found that the last portion of the benzoic acid was eliminated slowly.

Conjugation of Benzoic Acid with Glycine

One factor which remains undetermined is the rate with which the conjugation of benzoic acid with glycine takes place. Unfortunately no method could be found by which the speed of this combination could be determined. When sufficient glycine is supplied with ingested benzoic acid, the elimination of hippuric acid is practically the same as when hippuric acid itself is taken by mouth. Thus, the union of glycine with benzoic acid must proceed at a faster rate than the excretion of hippuric acid. Since the rate of the excretion of hippuric acid in the fasting state depends primarily on the amount of glycine synthesized and secondarily on the capacity of the kidney to eliminate hippuric acid, the actual conjugation of benzoic acid with glycine becomes a factor of minor importance. The site of this synthesis, conse-

quently, while still of theoretical interest loses in practical importance. While the evidence seems rather convincing that in the dog the synthesis of hippuric acid is confined solely to the kidney, it must be remembered that this animal is strikingly deficient in effecting this synthesis. Whether the synthetic power to combine glycine with benzoic acid is possessed by organs other than the kidney in man and those animals that produce much hippuric acid is a problem which requires further experimental work.

TABLE VII

Conjugation of Benzoic Acid in Fasting Showing Constant Rate of Hippuric Acid Excretion

Subject Q.

Time	Urine volume	Benzoic acid excreted as		
		Free	Hippuric acid	Glycuronic acid monobenzoate
	cc.	gm.	gm.	gm.
8 00-10 00 a.m.	30		0.04	0 05
10.00-12 00*	150	0.03	1.65	0.14
12 00- 2.00 p.m.	235	0.04	1.89	0.17
2.00- 4.00	140	0.03	1.93	0.28
4.00- 6 00	110	0.01	1.65	0.27
6 00- 8 00	110	0.01	1.55	0.27
8.00- 8.00 a.m.	275	0.08	3.10	0.65

* Took 10 gm. of benzoic acid at 10.00 a.m., and an additional 5.0 gm. at 11.00 a.m. Subject became nauseated in the afternoon, vomited once, and later developed a severe headache. Recovery was complete the following morning.

Conjugation of Benzoic Acid with Glycuronic Acid

The present study has thrown little light on the relationship of the conjugation of benzoic acid with glycine and with glycuronic acid. It was found that when a sufficiently large dose of benzoic acid was given so that the body's ability to synthesize glycine was strained, a portion of the benzoic acid (about 10 to 12 per cent) appeared in the urine combined with glycuronic acid. See Tables VII and VIII. It should be recalled that the writer has previously found that when a moderate dose of glycuronic acid monobenzoate is taken by mouth, none of the substance is excreted in the urine; the benzoic acid is eliminated as hippuric acid and the glycuronic

acid is presumably oxidized. It seems therefore reasonable to suppose that the amount of benzoic acid originally combined with glycuronic acid may be considerably larger than is indicated by the ultimate excretion of glycuronic acid monobenzoate. This is further suggested by the fact that when caffeine in the form of coffee or theobromine was taken with the benzoic acid, the reducing power of the urine appeared more pronounced.

It should be emphasized that glycuronic acid monobenzoate is much more labile than hippuric acid especially in alkaline solution, and therefore a urine containing glycuronic acid if allowed to become alkaline will contain free benzoic acid due to the resulting

TABLE VIII

Conjugation of Benzoic Acid with Glycine and with Glycuronic Acid
Subject W.

Time	Urine volume	Benzoic acid excreted as	
		Hippuric acid	Glycuronic acid monobenzoate
	cc.	gm.	gm.
8.00*– 9.00 a.m.	100	0.75	0.15
9.00 –10.00	174	1.06	0.17
10.00 –11.00	275	1.15	0.16
11.00 –12.00	60	1.10	0.12
12.00 – 1.00 p.m.	70	1.03	0.11
1.00 – 2.00	47	0.76	0.09

* Took 8.0 gm. of benzoic acid at 8.00 a.m.

hydrolysis. Perhaps much of the confusion existing in the literature on the problem of the excretion of free benzoic acid has been caused by glycuronic acid monobenzoate, the presence of which was not recognized.

While the conjugation of benzoic acid with glycuronic acid is far less important from the quantitative point of view than the conjugation with glycine, it must be recognized that a portion of the benzoic acid can be and is excreted as glycuronic acid monobenzoate and furthermore that this conjugation may be of far greater physiological importance than the amount excreted would indicate. There is furthermore, as will be discussed later, a possibility that this conjugation may be definitely disturbed in hepatic pathology.

Conjugation and Excretion of Benzoic Acid in Cases of Liver and Kidney Pathology

The use of sodium benzoate as a test for kidney function has never come into common use because it entailed a rather cumbersome analytical method for the determination of hippuric acid, and the results obtained showed little more than could be ascertained by simpler clinical procedures. It is not the purpose of this paper to discuss the clinical significance of the synthesis of hippuric acid, but rather to inspect the clinical material in order to secure additional information concerning the physiological processes involved in the handling of foreign substances such as benzoic acid.

The clinical studies on sodium benzoate emphasize how essential it is to have a thorough understanding of the response of the normal individual before it can be applied to a pathological condition. The test was developed as the result of Bunge and Schmiedeberg's experiments demonstrating that the excised kidney of the dog when perfused with benzoic acid and glycine produced hippuric acid. The synthesis of hippuric acid has since been generally regarded as a function of the kidney, and therefore it is commonly assumed that any impairment of the kidney should be reflected in a change in the conjugation of benzoic acid with glycine. As has already been stated, it is just this step in the mechanism which cannot be determined. When the dose of benzoic acid is relatively large and no exogenous glycine supplied, the test merely determines the rate of the synthesis of glycine; while if extra glycine is added, it measures the capacity of the kidney to excrete hippuric acid. As the synthesis of glycine is effected presumably by the liver, a determination of the maximum production as measured by the output of hippuric acid in the fasting state may perhaps show variations in hepatic disorders. Some clinical evidence has been found that there may be a decrease in the synthesis of glycine in certain diseases of the liver. Previous researches as those of Delprat and Whipple (52), and of Lackner, Levinson, and Morse (53), which are frequently quoted as proof that liver damage influences the synthesis of hippuric acid, are of doubtful value because these authors working with dogs failed to consider the conjugation of benzoic acid with glucuronic acid. As a matter of fact definite changes in the production of glucuronic acid monobenzoate even in humans may occur as the result of

pathological changes in the liver. This can be illustrated by a patient who had a markedly enlarged liver and fairly typical signs of multiple neuritis, probably alcoholic in origin. When she received 5.0 gm. of benzoic acid by mouth, she excreted in 5.5 hours, 0.65 gm. of benzoic acid conjugated with glycuronic acid, and 2.5 gm. combined with glycine. Several months later when her clinical condition had shown great improvement, she no longer excreted glycuronic acid when the same quantity of benzoic acid was again given. Such a finding cannot be explained at present because of the lack of information concerning the physiological function of glycuronic acid.

The power of the kidney to eliminate hippuric acid may perhaps serve as a valuable test to determine the efficiency of this organ. Instead of determining the rate of excretion as Kingsbury and Swanson (54) have done, a more sensitive index may perhaps be found in the maximum hourly output of hippuric acid, since it appears to be fairly constant in normal individuals. This and related problems will be studied clinically and reported later.

SUMMARY

1. Quinic acid when ingested gives rise to a prolonged excretion of hippuric acid. Similar results are obtained after eating prunes, indicating that the precursor of benzoic acid in this and perhaps other fruits is quinic acid.

2. The most important factor in the excretion of hippuric acid following the ingestion of benzoic acid in the fasting state is the rate at which glycine is synthesized by the body. If the amount of benzoate taken is sufficiently large, the maximum rate of the synthesis of glycine can be determined, and it appears that it is a constant depending on the size of the individual.

3. The function of glycine in the body is discussed. The theory is presented that the decrease in uric acid excretion following the administration of sodium benzoate is due to the effect upon the kidney of a relative deficiency of glycine.

4. Glycuronic acid monobenzoate is excreted in small but definite amounts following moderately large doses of benzoic acid.

5. No method was found for determining the actual rate with which glycine is conjugated with benzoic acid.

6. The possible clinical application of studies on the conjugation of benzoic acid is briefly discussed.

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A CRITICAL STUDY OF THE ANTIMONY TRICHLORIDE COLOR TEST FOR VITAMIN A

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The recent criticism of the antimony trichloride color reaction as a specific test for vitamin A, by Hawk (1), and Jones *et al.* (2), while others still accept the test as at least qualitative is responsible, in part at least, for this study of the antimony trichloride color test. In 1925, Rosenheim and Drummond (3) showed that arsenic trichloride, dimethyl sulfate, trichloroacetic acid, acetyl chloride, and some inorganic dehydrating agents such as sulfuric acid and phosphorus trichloride give a deep blue color with cod liver oil. In 1926, Carr and Price (4) suggested a reaction based on the use of antimony trichloride, but failed to show any quantitative relation between the color test and biological assay. Wokes and Willimott (5) suggested the use of a saturated solution of antimony trichloride, and also that the solution of the oil should be made the same day as it was to be used.

Concerning the specificity of the test the following references will give some idea of the variance of opinion with regard to its accuracy. Jones, Briod, Arzoomanian, and Christiansen (2) compared the biological test with the Wokes method and found the results incompatible. Norris and Danielson (6) claim that the color test checks within reasonable limits with the biological assay. Von Euler, Rydbom, and Hellstrom (7) modified the method by determining the greatest dilution at which the blue color was just visible. With this method, results closely agreeing with the animal testing methods were obtained. Ahmad and Drummond (8) found that results of animal tests agree within reasonable limits with those of the colorimetric method, although they make no claim that the color reaction is specific. Evers (9) found that the quantity of oil taken influenced the final color.

By adding an inactive oil, *e.g.* peanut oil, he found his results to be more uniform. Hawk (1) claims that cod liver oil exposed to air or other similar treatments showed a deeper blue color with antimony trichloride than oil kept in the dark. This and other observations caused Hawk to question the validity of the antimony trichloride test for vitamin A. Drummond (10) could not, on the other hand, confirm this observation of Hawk. Norris and Church (11) have shown that the test must be controlled with respect to the temperature, time, and concentration of the reagents.

Von Euler and Rydbom (12) have defined the unit quantity of vitamin A as a substance sustaining growth promotion for a period (8 weeks) on a minimal dosage of 0.05 mg. per day. Drummond (13) has pointed out that in feeding tests a test is really made to determine whether all the deficiencies of the diet are made good by the supplement, and hence there must be considerable possible error and variation in these biological assays.

Wokes (14) (with one of us (W. R. B.)) pointed out that with cod liver oil and antimony trichloride, two bands are produced, one between 475 and 482 $m\mu$ and the other between 535 and 550 $m\mu$. There is also a sharply defined band at about 614 $m\mu$ which fades and after several minutes a second band is produced at 528 $m\mu$. Concomitant with this fading there is a change in color of the solution from blue to red.

There has apparently been no systematic study of this color reaction in which the absorption spectra of the solutions were determined, the original investigators and most of the later workers using the Lovibond tintometer or some form of colorimeter rather than a spectrophotometer. In the present work a Bausch and Lomb spectrophotometer was used. This instrument had attached to it a modified Duboscq colorimeter which permitted the rapid introduction of solutions into the optical path, the rapid change of cell thickness, and the use of a convenient all glass cell. The construction of this spectrophotometer permitted either a qualitative examination over a wide portion of the spectrum to determine the position of the band or a quantitative determination, within a narrow portion of the spectrum, of the intensity of the band. Observations on a number of solutions showed that the blue solutions produced a band in the red

portion of the spectrum which was not always in the same place but varied between two different wave-length positions—one at $578\ m\mu$ and the other at $610\ m\mu$. The separate identity of these bands could be shown in some solutions in which both bands could be observed at the same time, while in other solutions either one or the other appeared. The maxima of both of these bands were obtained in about the same time interval, 20 to 40 seconds at 25° . However, since readings could be started within 5 seconds after the solutions were mixed together and continued at 5 second intervals thereafter, no difficulty was experienced in observing the maxima and in actual practice greater time intervals than 5 seconds were used.

Examination of the faded or red solutions showed the presence of two bands, one at $532\ m\mu$ and the other at $472\ m\mu$. On comparison of these results with those obtained from the blue solutions it was found that solutions exhibiting the $578\ m\mu$ band gave on fading the $472\ m\mu$ band and the solutions exhibiting the $608\ m\mu$ band gave on fading the $532\ m\mu$ band. Solutions showing both the 578 and $608\ m\mu$ bands gave both the 472 and $532\ m\mu$ bands. The earlier workers, using colorimetric methods, rather than spectrophotometric methods of observation were unable to distinguish other than slight differences in hue between these two solutions. Two possible explanations for the existence of these two series of bands are that two different substances are present, or that the conditions of concentration, temperature, or other substances present may cause a change in the molecular configuration of the color-forming substance. The available evidence seems to favor both of these explanations. By changing the concentration of solutions, changing the temperature, or by the introduction of certain chemicals an oil may be made to give either of the two bands, providing, of course, that some of the color-forming material is present. On the other hand carotene, the much discussed supposed precursor of vitamin A, gives with the antimony trichloride reagent a sharp band at $590\ m\mu$ which fades as do the cod liver oil bands to yield a band at $488\ m\mu$.¹ From these latter results and from a number of observations on other oils it seemed that of the two possible bands which might

¹ We are indebted to Doctor James H. C. Smith of the Carnegie Institution of Washington for a sample of carotene for these experiments.

be used to indicate the concentration of the vitamin A, the $608\text{ m}\mu$ band was to be preferred.

The optimum conditions of concentration for the production

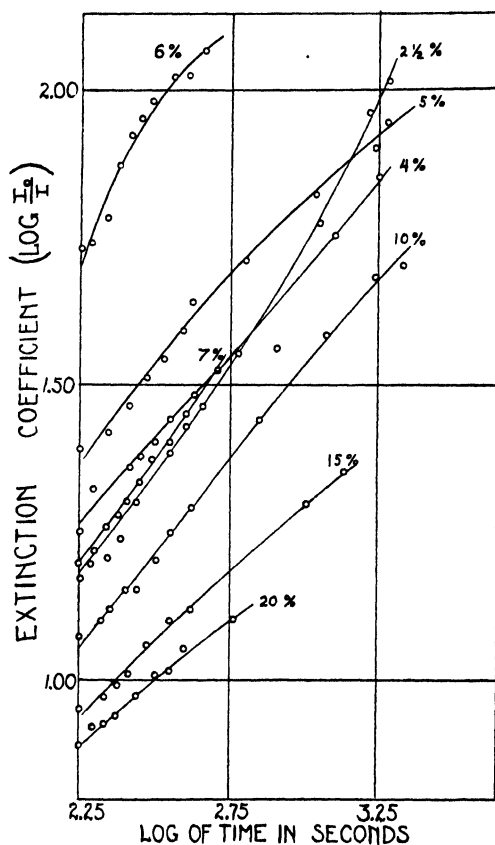


FIG. 1. Change of extinction coefficient values ($532\text{ m}\mu$ band) with time for various concentrations of cod liver oil. The concentration of SbCl_3 in these solutions was 14 per cent (unsaturated). The extinction coefficient values have been reduced to equivalent oil concentrations.

of the $608\text{ m}\mu$ band were determined by a series of tests on a few commercial samples of cod liver oil.

It was found that the best results were obtained by using a saturated solution of antimony trichloride in anhydrous chloro-

form. With a less than saturated solution of antimony trichloride, the absorption readings were not proportional to the amount of oil taken, without making a correction. The effect of concentration of SbCl_3 reagent can be seen in the accompanying graphs. In Fig. 1, a 14 per cent solution of SbCl_3 is used, and the spectro-

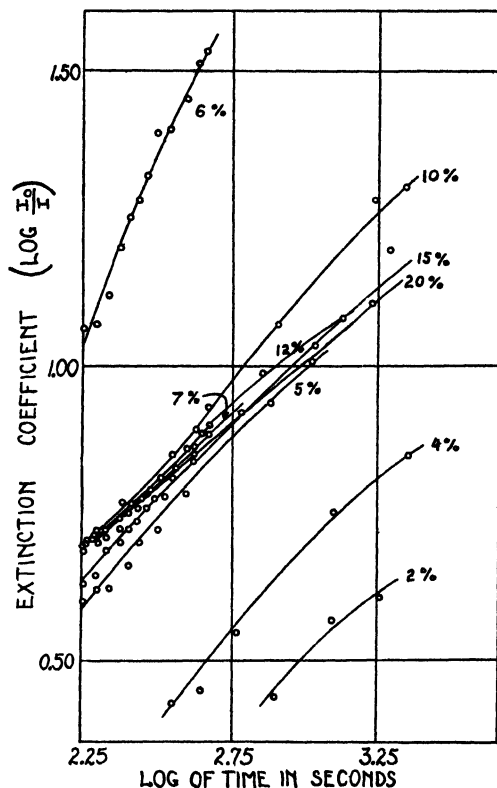


FIG. 2. Same as Fig. 1, except that the extinction coefficient values have been corrected by subtracting 0.40 before being reduced to equivalent oil concentrations.

photometric readings are changed to what they would be if a 10 per cent solution of oil had been used, and a cell thickness of 4 cm. In Fig. 2, a correction (empirical) has been made by subtracting 0.40 from the spectrophotometric readings before changing the concentrations to 10 per cent. With this correction,

the values check very closely with each other, except for solutions containing 6 per cent or less of oil. With a saturated solution of SbCl_3 , the readings are proportional to the concentration of the oil. These readings were made on the $532\text{ m}\mu$ band. Figs. 3 and 4 show the effect of varying the concentration of the SbCl_3 reagent on the $608\text{ m}\mu$ absorption band. Again, with saturated solutions

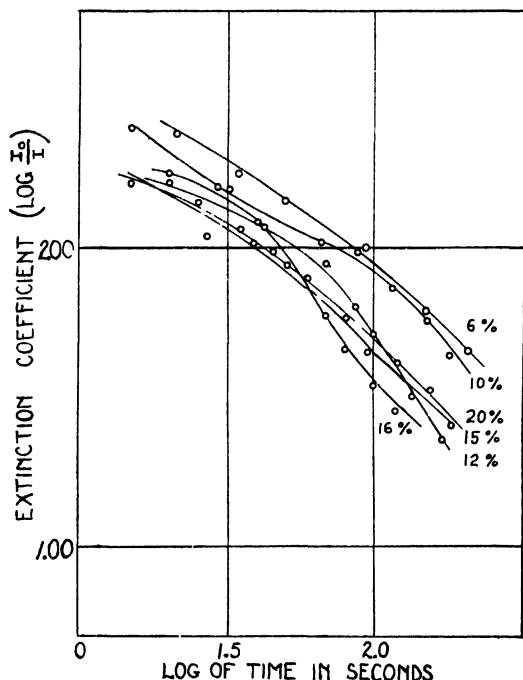


FIG. 3. Change of extinction coefficient values ($608\text{ m}\mu$ band) with time, for various concentrations of cod liver oil. The concentration of SbCl_3 in these solutions was 18.5 per cent (saturated). The extinction coefficient values have been reduced to equivalent oil concentrations.

of SbCl_3 , the values of the extinction coefficient, with the exception of those below 6 per cent, are proportional to the concentration of the oil used. With less than saturated solutions of SbCl_3 , the readings are not proportional to the concentration of the oil.

The saturated solution of antimony trichloride in chloroform was prepared in the following manner. Anhydrous antimony

trichloride was purified by the distillation of the c. p. product in a current of dry hydrogen chloride gas in an all glass apparatus. The distillate was collected in a dry, round-bottomed liter flask. Anhydrous chloroform was prepared by washing the c. p. product with water to remove the alcohol, dried over calcium chloride, and finally dried with sodium. The chloroform was poured off

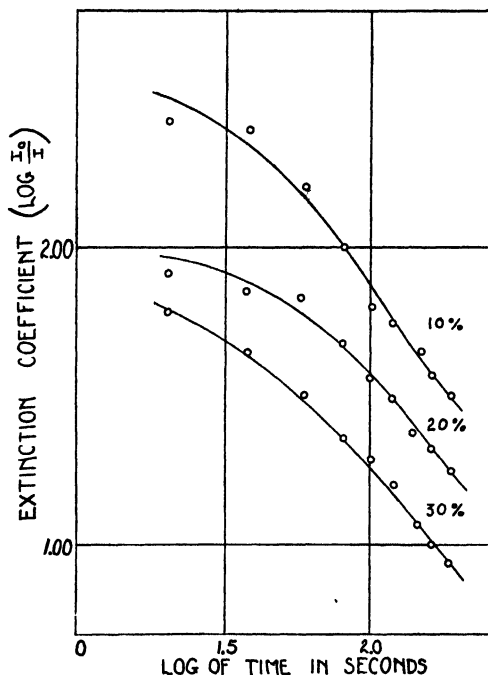


FIG. 4. Change of extinction coefficient values (608 $m\mu$ band) with time for various concentrations of cod liver oil. The concentration of $SbCl_3$ in these solutions was 16 per cent (unsaturated). The extinction coefficient values have been reduced to equivalent oil concentrations.

from the sodium and distilled (b.p. 61.5°). The solution of the reagent was prepared by adding the anhydrous chloroform to the anhydrous antimony trichloride, and refluxed until the antimony trichloride was all melted (m.p. 73°). The mixture was shaken gently and then allowed to cool, whereby the excess antimony trichloride crystallized out. By this method, a saturated solution

of antimony trichloride in chloroform (about 18.5 per cent by weight) could be quickly made. This solution becomes yellow on standing and should therefore be renewed frequently.

The concentration of the oil used determines whether the spectroscopic readings are proportional to the amount of oil taken. It was found that by using a saturated solution of antimony trichloride and a solution of cod liver oil of such a strength that only the 608 $m\mu$ band is produced that the concentration of the oil is proportional to the reading of the 608 $m\mu$ band 20 seconds after mixing. The time, 20 seconds, was chosen because the maximum

TABLE I

Effect of Changing the Concentration of the Reagent or Oil on the Spectroscopic Readings

SbCl ₃	Cod liver oil	Cod liver oil solution	Band produced	
			608 $m\mu$	578 $m\mu$
<i>per cent</i>	<i>per cent</i>	<i>cc.</i>		
9.5	20	0.1-1.2		Present
9.5	40, 60	1.0		"
13.0	20	0.1-0.5	Present	
16.5	1	0.1-0.5, 4		
16.5	2	1, 2		
16.5	2	5	Present	
16.5	10, 20, 30	0.5	"	
16.5	40, 50	0.5	"	Present
16.5	60, 70, 80, 90, 100	0.5		"
Saturated	2-30	0.5	Present	
"	40, 50	0.5	"	Present
"	50-100	0.5		"

absorption generally occurs then. If a more concentrated solution of oil is used, the 578 $m\mu$ band is produced. In the particular oils tested, the 608 $m\mu$ and 532 $m\mu$ readings for a 6 per cent or less solution of oil are at variance with the expected values.

The procedure adopted for the analysis of oils is as follows: Place 5 cc. of saturated antimony trichloride solution in a spectrophotometric cell. Add 1 drop of acetic anhydride (to react with hydrochloric acid, water, etc., present). Add 0.5 cc. of a chloroform solution of the oil to be tested, so that it forms a layer on top of the antimony trichloride reagent. Mix by shaking, and at

exactly 20 seconds after mixing, the intensity of the 608 $m\mu$ band is observed. Then a reading is made at 578 $m\mu$, to note if there is a band there. If a band is present at 578 $m\mu$, the oil solution is too concentrated, and a more dilute solution should be used.

TABLE II
Relationship between Bands at 608 $m\mu$ and 532 $m\mu$

Observation No.	Cod liver oil	Extinction coefficient		Ratio 608 $m\mu$ 532 $m\mu$	Mean	Probable error
		608 $m\mu$ (20 sec.)	532 $m\mu$ (5 min.)			
	<i>per cent</i>					
1	10	1.50	0.48	3.12	2.84	± 0.09
2	10	1.75	0.70	2.50		
3	10	1.65	0.55	3.00		
4	10	0.95	0.45	2.11		
5	10	1.12	0.36	3.11		
6	10	1.27	0.39	3.28		
7	10	1.25	0.55	2.28		
8	10	1.76	0.62	2.84		
9	7	1.59	0.50	3.18		
10	13	1.67	0.57	2.93		

TABLE III
Relationship between Bands at 578 $m\mu$ and 472 $m\mu$

Observation No.	Cod liver oil	Extinction coefficient		Ratio 578 $m\mu$ 472 $m\mu$	Mean	Probable error
		578 $m\mu$ (maximum)	472 $m\mu$ (5 min.)			
	<i>per cent</i>					
11	40	1.82	1.65	1.10	1.26	± 0.03
12	50	0.90	0.64	1.41		
13	60	0.94	0.77	1.22		
14	70	1.32	1.10	1.20		
15	80	1.10	0.76	1.44		
16	90	1.62	1.35	1.20		
17	100	1.85	1.48	1.25		

In order to check the results, another solution of oil of a different concentration is made and observed. The readings of the 608 $m\mu$ band should be proportional to the amount of oil used. Since a 10 per cent solution of oil usually gives good results, it is customary to try the 10 per cent first. If the extinction coefficient

of the 608 $m\mu$ band for 2 cm. cell thickness is less than 1.50 to 1.75, the check solution may be 20 per cent. If the reading is greater than 1.75, but less than 2.00 to 2.50, a 15 per cent solution is usually correct for the check. If greater than 2.00 to 2.50, the check solution should be 7 or 8 per cent. If the 10 per cent solution gives a 578 $m\mu$ band, weaker solutions must be used.

The effect of changing the concentration of the reagent and of the oil is shown in Table I. It was found that either, or both, the 578 $m\mu$ or 608 $m\mu$ bands could be produced.

When the 608 $m\mu$ band fades, the 532 $m\mu$ band appears. When the 578 $m\mu$ band fades, the 472 $m\mu$ band appears. Tables II and III show that some relation exists between these two sets of bands. A similar relationship can be shown between the 578 $m\mu$ band and the 472 $m\mu$ band.

The probable error is calculated from the formula

$$\text{Probable error}^* = \frac{0.8453 \Sigma (+v)}{n \sqrt{n-1}}$$

where $\Sigma (+v)$ is the sum of all the deviations from the mean without regard to sign, and n is the number of values.

For low temperature work, the saturated solution of antimony trichloride had to be diluted to 0.5 concentrated, and a little extra acetic anhydride added to keep the antimony trichloride in solution.

The following experiments were tried.

Experiment a—5 cc. of saturated solution (18.5 per cent) of SbCl_3 , 5 cc. of CHCl_3 , 4 drops of acetic anhydride, and 1 cc. of 6 per cent cod liver oil.

This mixture at 0° gave bands at 578 and 608 $m\mu$. These bands were narrow at first, and then they spread out. The 578 $m\mu$ band faded much more rapidly than the 608, and the solution was still blue at the end of 15 minutes. The solution gradually became colorless and after 60 minutes it began to turn pink (at room temperature this occurred in about 8 minutes).

Experiment b—With 1 cc. of 3 per cent oil, weak bands were produced at 578 and 608 $m\mu$ at 0°.

* Mellor, J. W., Higher mathematics for students of chemistry and physics, London, 524-531 (1913).

Experiment c—With 1 cc. of 5 per cent oil at 0°, a rather broad weak band is produced at 578, and a strong narrow band at 608 $m\mu$.

Experiment d—With 1 cc. of 5 per cent oil at -5°, there were no bands at first, in 2 minutes one at 608 developed, and in 2½ to 3 minutes a 578 $m\mu$ band developed. The 608 $m\mu$ band became more intense than the 578. The band at 608 $m\mu$ lasted about 35 minutes, and the one at 578 about 10 minutes.

Experiment e—These solutions (Experiments a, b, c, and d) had to be taken out of the cold brine to be read, thus causing them to warm up somewhat and hastening the fading. Another sample was kept in ice for 1¼ hours and then read. The 608 $m\mu$ band was still fairly strong, but the 578 $m\mu$ band was absent.

SUMMARY

It has been shown that the $SbCl_3$ color solutions in the test for vitamin A may have two different absorption bands, one at 578 $m\mu$ and the other at 608 $m\mu$. Both of these bands fade and the respective solutions develop new bands at 472 and 532 $m\mu$.

Conditions of concentration have been determined by which only one of the two bands is produced (608 $m\mu$). Cod liver oils when observed under these conditions yield extinction coefficient values of the 608 $m\mu$ band which are proportional to the concentration of the oil. There appears to be a definite relation between the extinction coefficient values of the blue solution (608 and 578 $m\mu$ bands) and the faded or red solutions (532 and 472 $m\mu$ bands).

Data will be presented in a subsequent paper on the comparisons of results obtained by this method with those obtained by biological assay.

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DIMINUTION IN CHLORIDE MEASUREMENT AFTER DRYING BLOOD AND TISSUES

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In determining the concentration of chloride in tissues, we have found that the amount of chloride recovered from the material analyzed after drying was always less than the amount recovered from the material analyzed in the wet state, the difference amounting at the maximum to 31 per cent. Our analyses have been made on blood, blood serum, voluntary muscle, heart, and liver. Because of the greater concentration of chloride present and the greater facility in analyzing a fluid tissue, blood has been used chiefly for the study of the nature of this diminution. Our analyses have been made on different specimens of essentially normal tissues but since in the analyses of any given tissue we have observed little individual variation in our particular series from the mean, a compilation of our data, such as is shown in Table I, will suffice to illustrate the diminution of chloride measured after drying the various tissues studied.

Although in most of our determinations we have used the Volhard titration according to the Wilson and Ball modification (1) of the Van Slyke method (2), many analyses on blood were made and similar results obtained when larger amounts of material were used and chloride was determined gravimetrically by the open Carius (3) and the Thompson-Oakdale (4) methods. In no variations of the Van Slyke method tried, such as the addition of increasing amounts of HNO_3 or the introduction of KMnO_4 during digestion, have the recoveries varied by as much as 1 per cent from results obtained with the original method.

To determine the length of time required for $\text{AgNO}_3\text{-HNO}_3$ digestion in the water bath with the Wilson and Ball procedure, titrations were made after periods of digestion varying from $1\frac{1}{2}$ to 20 hours. In these analyses our maximum recoveries were obtained in less than 2 hours for digestion of wet blood and 6 hours for digestion of dried blood and other wet and dried tissues. These time intervals have been used in our standard technique.

TABLE I
Mean Values of Analyses of Wet and Dried Tissues

Calculated on a wet tissue basis.

Material	No. of determinations	Method	Mean Cl content of wet samples	Mean Cl content of dried samples	Mean Cl difference
			<i>m.-eq. per l.</i>	<i>m.-eq. per l.</i>	<i>m.-eq. per l.</i>
Human serum	20	Wilson-Ball	101.9	98.3	3.6
“ blood	20	“	76.5	58.8	17.7
Ox serum	20	“	99.0	93.0	6.0
“ blood	20	“	78.8	67.5	11.3
“ “	22	Open Carius (gravimetric)	82.3	70.1	12.2
“ “	7	Thompson-Oakdale	76.1	68.1	8.1
Lamb blood	12	Wilson-Ball	80.0	68.3	11.7
“ “	8	Open Carius (gravimetric)	84.9	72.0	12.9
Dog serum	10	Wilson-Ball	106.2	101.1	5.1
			<i>m.-eq. per kg.</i>	<i>m.-eq. per kg.</i>	<i>m.-eq. per kg.</i>
Beef muscle	24	Wilson-Ball	19.3	14.8	4.5
“ liver	12	“	28.8	25.0	3.8
“ heart	6	“	27.6	19.0	8.6

In the analyses of liver and muscle, the material was ground several times in a meat grinder and the resulting hash was thoroughly mixed. From 2 to 3 gm. of this hash were weighed in stoppered Erlenmeyer flasks. Chloride analyses on these samples have been expressed in terms of milli-equivalents per kilo of wet tissue. In the analyses summarized in Table I, drying was accomplished by placing the blood and blood serum for 24 hours, and the muscle, heart, and liver tissues for 48 hours, in a drying oven

regulated between 100–110°. The chloride analyses given in the tables represent the average of triplicate determinations for tissues and of duplicate determinations for blood.

Experiments on the Nature of the Diminution of Chloride Recovery after Drying

The effect of duration of drying was tested on one specimen of serum. Maximum diminution of chloride recovery after drying this serum at 100° was obtained within 5 hours. Drying the same serum for periods as long as 105 hours did not alter this diminution. Blood was dried for a period of 7 days to constant weight at 25° in a desiccator containing P_2O_5 and through which was passed a continuous stream of air dried in H_2SO_4 . Analyses of blood dried in this manner revealed a decrease in chloride recovery similar to that obtained from samples of the same blood dried at 100° in a drying oven. To eliminate the possibility that the diminution of chloride observed was due to volatile chloride given off during the drying process, the distillate during drying was collected and analyzed for both organic and inorganic chloride. No chloride could be recovered from the distillate.

Factors Influencing the Diminution of Chloride Recovery after Drying

When known amounts of KCl or NaCl in concentrations from 5 to 400 milli-equivalents per liter were added to blood, theoretical recoveries were obtained on the wet analyses; however, with dried blood to which NaCl or KCl in concentrations from about 40 to 400 milli-equivalents per liter had been added before drying, the decrease in the chloride measurement was approximately doubled. In Table II is given one of the several experiments in which known quantities of chloride were added to blood.

An additional factor, which appeared to influence the decrease in chloride measurements due to drying blood with added amounts of NaCl or KCl, was the length of time during which the NaCl or KCl solutions were in contact with the blood before being placed in the drying oven. When blood with added chloride was permitted to stand at room temperature for 2 hours before drying, a greater diminution in chloride measurement was observed. Thus in one typical experiment in which 57.0×10^{-6} equivalents of KCl

were added respectively to each 1 ml. sample of blood, the chloride difference after standing at room temperature for 10 minutes before being placed in the drying oven was 12.1×10^{-6} equivalents; after standing for 120 minutes this difference was increased to 25.4×10^{-6} equivalents.

The analyses thus far described were made directly on the dried residues from materials which had been pipetted or weighed either into test-tubes or flasks before drying. It was desired to obtain the chloride analysis of materials which had been pulverized after

TABLE II
Varying Amounts of KCl Added to Ox Blood

Material	Cl analyses of wet samples	Cl analyses of dried samples	Cl difference
	<i>m.-eq. per l.</i>	<i>m.-eq. per l.</i>	<i>m.-eq. per l.</i>
Blood	80.5	66.6	13.9
“ + KCl	85.6	75.4	10.2
	91.2	79.3	11.9
	95.9	83.0	12.9
	101.0	89.3	11.7
	106.1	94.0	12.1
	111.4	100.2	11.2
	121.6	106.1	15.5
	131.9	110.2	21.7
	157.5	133.1	24.4
	183.1	159.0	24.1
	208.8	184.3	24.5
	285.7	265.3	20.4
	388.3	363.4	24.9
	490.9	470.1	20.8

drying. Accordingly a portion of blood was dried, ground in a mortar, and analyses were made on this pulverized material. The diminution of chloride measurement in pulverized dried blood was less than one-half of that observed in the same dried blood which had not been pulverized.

Chloride Analyses after Allowing Dried Tissues to Stand in Contact with Water

Experiments were carried out to study the effect of allowing the dried tissues to stand in contact with water. It was found that if

the dried tissues were covered with a sufficient quantity of water for an appropriate period of time, the chloride recoveries were

TABLE III

Cl Analyses after Allowing Dried Tissues to Stand in Contact with Water

The values are calculated on a wet sample basis.

Material (1 ml. samples of blood, 2 to 3 gm. samples of tissue)	Method	Cl analyses of wet samples	Cl analyses of dried samples	Cl analyses of dried samples in contact with H ₂ O
		m.-eq. per l.	m.-eq. per l.	m.-eq. per l.
Ox Blood 7	Wilson-Ball	73.4	60.1	72.9
" " 9	"	77.8	67.5	78.5
" " 11	Open Carius (gravimetric)	81.5	71.6	82.5
" " 12	Open Carius (gravimetric)	83.0	72.0	84.0
Lamb Blood 5	Wilson-Ball	80.0	68.3	80.1
Human "	"	79.2	65.4	79.9
		m.-eq. per kg.	m.-eq. per kg.	m.-eq. per kg.
Beef Muscle 11	Wilson-Ball	17.3	14.2	16.2
" " 12	"	20.5	15.3	19.8
" " 13	"	24.7	17.3	23.2
" " 14	"	13.6	12.1	14.3
" Liver 2	"	25.5	21.0	25.1
" " 3	"	32.0	28.9	32.4
" heart	"	27.6	19.0	24.9
		μ eq.*	μ eq.	μ eq.
Ox Blood 10				
1 ml. blood	Wilson-Ball	78.8	67.5	78.5
1 " " + 1 ml. NaCl (10.5 μ eq.)	"	88.9	77.7	88.7
1 ml. blood + 1 ml. NaCl (18.1 μ eq.)	"	95.3	83.2	96.2
1 ml. blood + 1 ml. NaCl (37.6 μ eq.)	"	115.5	99.1	110.6
1 ml. blood + 1 ml. NaCl (74.4 μ eq.)	"	156.8	139.0	153.7

* μ eq. = 10^{-6} eq.

practically the same as those from the wet specimens. In Table III are given our analyses of normal tissues, dried tissues, and dried tissues that had stood in water. With the Wilson and Ball method

and using 1 ml. samples of blood and 2 to 3 gm. samples of muscle and liver before drying, essentially complete recoveries of chloride were obtained after contact with 5 ml. of water for 3 hours. Similar recoveries were obtained with the open Carius gravimetric method using 5 ml. samples of blood.

Complete recovery of chloride was obtained in dried blood after contact with an aqueous solution of AgNO_3 for a period of more than 2 hours before digestion with HNO_3 . If HNO_3 was added during the 1st hour of contact with either water or an aqueous solution of AgNO_3 , the results obtained were lower than those for the original wet specimen.

When NaCl in concentrations from 10 to 75 milli-equivalents per liter was added to blood, these mixtures, dried and then kept in contact with water, gave essentially theoretical results as shown in Table III.

A Factor Responsible for Diminution of Chloride Measurement after Drying

In an effort to determine possible constituents of the blood which were responsible for the difference in chloride analysis after drying tissues, several experiments were made. It has been reported by others (5) that large amounts of sugar (of the order of 10 gm. of sugar to 0.1 gm. of NaCl) interfere with chloride determinations by the open Carius method, unless the sugar be oxidized by prolonged digestion with KMnO_4 . To determine whether sugar or urea in approximately the same concentration as is present in the blood interferes with chloride recoveries from dried samples, weighed amounts of NaCl were added to solutions of dextrose and of urea. The chloride recoveries from these solutions, either wet or dried, were theoretical. Chloride analyses of serum to which dextrose had been added in quantities of as much as 700 mg. of dextrose per 100 ml. of serum, showed no essential deviation from the chloride analyses on the original serum without added dextrose. From these experiments we believe that sugar and urea in blood are not present in sufficient quantities to interfere with the chloride recoveries after drying.

Chloride determinations made on protein-free filtrates of blood after tungstic acid precipitation, according to the Whitehorn method (6), were practically the same on both the wet and the

dried filtrates (Table IV). In addition, an ultrafiltrate was prepared from blood which had been placed in collodion sacs under a pressure of 200 mm. of Hg. The chloride analyses made of the dried ultrafiltrate were the same as those of the wet (Table IV).

In order to determine whether chloride was occluded by the drying of a pure protein and not released by $\text{AgNO}_3\text{-HNO}_3$ digestion, chloride analyses were made on a solution of reduced hemoglobin with added KCl. The reduced hemoglobin was prepared from base-free isoelectric crystals of horse hemoglobin (Stadie and Sunderman (7)). Analyses of this solution showed no differ-

TABLE IV .
Miscellaneous Analyses

The values are calculated on a wet sample basis.

Material	Cl analyses of wet samples	Cl analyses of dried samples	Cl difference	Cl analyses of dried samples in contact with H_2O
	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.
Blood filtrate (after tungstic acid precipitation)	87.0	87.0	0.0	
Ultrafiltrate of ox blood.....	82.1	82.3	+0.2	
Reduced hemoglobin + KCl solution.....	102.1	101.6	-0.5	
Solution I (gum acacia + NaCl) ..	133.1	132.5	-0.6	
" II (emulsion of gum acacia, olive oil, and NaCl).....	96.1	69.0	-27.1	95.4
Olive oil + NaCl.....	74.8	69.3	-5.5	71.2
" " + " 	49.9	42.1	-7.8	49.6
" " + " 	24.7	21.4	-3.3	24.8

ence of chloride on drying within the limit of the error of our measurements (Table IV).

To determine whether fats interfered with the chloride measurement after drying, analyses were made on the two following solutions. Solution I consisted of a 7 per cent gum acacia solution and added NaCl; Solution II, of an emulsion containing 10 per cent gum acacia, 15 per cent olive oil, and added NaCl. The results of the analyses on both the wet and dried specimens of these solutions are shown in Table IV. No difference of chloride measurement was observed in the analyses of dried Solution I, but there

was a 27 per cent diminution of chloride measured in dried Solution II. Dried specimens of Solution II when allowed to stand in contact with water for 3 hours gave a theoretical recovery.

Theoretical recovery of chloride was obtained in a solution of NaCl to which olive oil had been added. However, when the water was evaporated from the mixture of NaCl solution and olive oil by drying at 100° , the chloride recovery was less. Allowing such anhydrous samples to stand in contact with water before the $\text{AgNO}_3\text{-HNO}_3$ digestion gave complete recoveries. From these experiments we conclude that fat can be an interfering constituent in the Cl analysis of dried substances.

DISCUSSION

These experiments demonstrate the diminution in chloride measurement of blood and tissues dried at $100\text{--}110^{\circ}$ and digested by AgNO_3 and HNO_3 . They also demonstrate that measurements are obtained which are essentially the same as those of the original analyses of the blood and tissues in the wet state if the dried blood or tissues are allowed to stand in contact with water before digestion with AgNO_3 and HNO_3 . The experiments with chloride and olive oil suggest the possible importance of fats in this phenomenon. Our attention has been called to a recent publication of Morris and Morris (8) containing experiments identical in type with those which one of us (F. W. S.) conducted in the early stages of this study. The conclusion of Morris and Morris that volatile chloride is lost from blood during the period of drying at $100\text{--}110^{\circ}$ and is responsible for the diminution in the chloride recovered after drying is, in the light of our experiments, clearly incorrect. That traces of halogen distil off during digestion of blood and tissues with AgNO_3 and HNO_3 is suggested by studies which we have still in progress. However, the amounts of halide which we have recovered from such distillates have been entirely insufficient to account for the diminution of chloride measurement of blood and tissues after drying.

To obtain reliable results consistently for blood chlorides with the open Carius titrimetric procedure, Wilson and Ball added aqueous AgNO_3 before adding HNO_3 . We are able to confirm their findings that increased accuracy is secured in certain instances by the modification. Wilson and Ball believed that the

reason for their improvement might be the precipitation of AgCl before the precipitation of protein. Our studies suggest that fats (or fatty acids) may be a disturbing factor.

From studies now in progress it would appear that maximal recovery of chloride even from wet tissues, such as liver and muscle, is not obtained consistently by immediate digestion after the simple addition of both AgNO_3 and HNO_3 . We are examining methods of insuring maximal recovery of the chloride from such tissues.

CONCLUSIONS

The quantity of chloride recovered by usual analytical methods from dried samples of blood and tissues was less than that recovered from the wet samples of the same materials.

The quantity of chloride recovered from the dried samples which had been in contact with water for an appropriate period of time before AgNO_3 - HNO_3 digestion was practically the same as that recovered from the original wet samples.

Our experiments suggest that fats (or fatty acids) and the state of division of the material analyzed may be responsible for the technical difficulty encountered.

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ACETYL MONOSES

VII. THE ISOMERIC TRIACETYL-1-METHYL-*D*-RIBOSIDES

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Fischer, Bergmann, and Rabe¹ have observed that on "methylation" of 1-bromotriacetyl rhamnose, a triacetylmethyl-*l*-rhamnoside is obtained, characterized by possessing one acetyl group stable towards alkali. Dale² found that 1-bromotetracetyl-*D*-mannose behaved similarly and Levene and Wolfrom³ found the same peculiarity in the case of lyxose. These anomalous forms were referred to as γ forms. Levene and Wolfrom⁴ have found that the rate of hydrolysis of the methyl group in the cases of γ -tetracetylmethyl-*D*-mannoside and of γ -triacetylmethyl-*D*-lyxoside resembles that of the furanosides, but Haworth and co-workers⁵ have shown that in the cases of rhamnose and mannose, the glycoside possesses the pyranose structure. These authors concluded also that the stable acetyl group is situated on carbon atom 2. On the other hand, Freudenberg and Braun,⁶ on the basis of spectroscopic analysis, reached the conclusion that the substance was not a true methylglycoside but that its carbon atoms 1 and 2 were linked to a methylated orthoacetic acid residue. Haworth and coworkers evolved a similar theory and structure independently and practically simultaneously.

¹ Fischer, E., Bergmann, M., and Rabe, A., *Ber. chem. Ges.*, **53**, 2362 (1920).

² Dale, J. K., *J. Am. Chem. Soc.*, **46**, 1046 (1924).

³ Levene, P. A., and Wolfrom, M. L., *J. Biol. Chem.*, **78**, 525 (1928).

⁴ Levene, P. A., and Wolfrom, M. L., *J. Biol. Chem.*, **79**, 471 (1928).

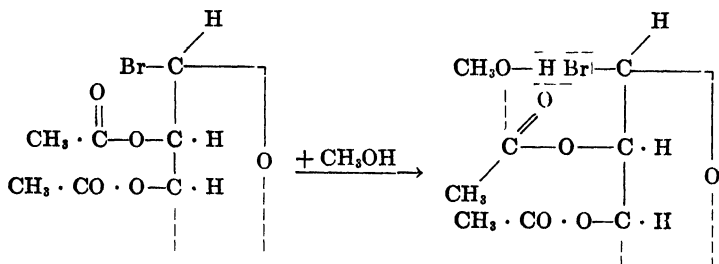
⁵ Haworth, W. N., Hirst, E. L., and Miller, E. J., *J. Chem. Soc.*, 2469 (1929). Bott, H. G., Haworth, W. N., and Hirst, E. L., *J. Chem. Soc.*, 1395 (1930).

⁶ Freudenberg, K., and Braun, E., *Naturwissenschaften*, **18**, 393 (1930).

As yet it is not known whether the peculiarity observed in these three sugars is common to all sugars having the hydroxyls of carbon atoms 2 and 3 in the *cis* position and because of this uncertainty the behavior of ribose has been made the subject of the present investigation.

It was found that 1-bromotriacetyl-*d*-ribose behaved similarly to the bromoacetyl derivatives of the other three sugars in forming a γ -triacetylmethyl-*d*-riboside which was obtained in crystalline form, with a m.p. of 77–78° and having $[\alpha]_D^{20} = +2.4^\circ$ (in chloroform). The methyl group was hydrolyzed by dilute acids as readily as in the other γ -acetylmethylmonosides, but hydrolysis by alkali only revealed the presence of two acetyl groups. The substance was distinctly different in its properties from the triacetylmethyl-*d*-riboside obtained on acetylation of methyl-*d*-riboside, which acted as a normal, pyranose derivative.

Assuming that the formation of derivatives of orthoacetic acid is a common property of sugars having the hydroxyls of carbon atoms 2 and 3 in the *cis* position, this behavior may possibly be ascribed to the bromine atom of the bromoacetyl derivative of *d*-ribose having the α -configuration, and of those of *l*-rhamnose, *d*-lyxose, and *d*-mannose having the β -configuration (*i.e.*, the functional groups of carbon atoms 1 and 2 are also in the *cis* position).



EXPERIMENTAL

Preparation of Tetracetyl Ribose—5 gm. of crystalline ribose were dissolved in a mixture of 19 cc. of acetic anhydride and 25 cc. of pyridine and the resulting solution was kept at 0° overnight. It was then poured onto finely crushed ice, with vigorous stirring, and the crystalline precipitate filtered off. On standing overnight at 0° the aqueous mother liquor deposited a further crop of

crystalline material. The aqueous solution was now extracted three times with chloroform and the chloroform extract washed successively with ice-cold dilute sulfuric acid, ice-cold dilute sodium bicarbonate solution, and finally with ice water until neutral. It was then dried by means of anhydrous sodium sulfate, filtered, and the filtrate evaporated to a thick syrup. This was dissolved in 95 per cent ethyl alcohol and on nucleating and cooling, deposited crystalline tetracetyl ribose.

The total yield (after one recrystallization from 95 per cent ethyl alcohol) was 7.5 gm.; m.p., 110°.

Its rotation was

$$[\alpha]_D^{25} = \frac{-3.08^\circ \times 100}{2 \times 2.960} = -52.0^\circ \text{ (in chloroform)}$$

The substance had the following composition.

3.500 mg. substance: 6.305 mg. CO₂ and 1.830 mg. H₂O.

C₁₈H₁₈O₈. Calculated. C 49.04, H 5.7

Found. "49.12, " 5.9

Preparation of Crystalline Bromotriacetyl Ribose—5 gm. of finely powdered tetracetyl ribose were mixed with 25 cc. of glacial acetic acid containing 40 per cent of dry hydrogen bromide. The resulting solution was allowed to stand at room temperature for 60 minutes, after which the hydrogen bromide gas was removed under diminished pressure at room temperature. The solution was then diluted with 100 cc. of toluene and evaporated to a thick syrup under diminished pressure at 35°. Two further portions of 50 cc. of toluene were run in and evaporated off. This syrup was now dissolved in 50 cc. of benzene and the solution was evaporated to a thick syrup. Traces of solvent were removed at high vacuum at 35°.

The resulting thick, very pale yellow syrup was dissolved in the minimum of cold dry ether, a further 5 cc. of ether were added, and then petroleum ether was added to incipient turbidity. A little charcoal was added and the mixture shaken and filtered on a fluted filter, the filtrate obtained being absolutely colorless.

On cooling in ice and scratching vigorously, crystallization immediately set in. Yield, 3.1 to 3.2 gm. of colorless crystals; m.p., 96°.

Its rotation was

$$[\alpha]_D^{25} = \frac{-7.55^\circ \times 100}{2 \times 1.804} = -209.3^\circ \text{ (in chloroform)}$$

The substance had the following composition.

8.150 mg. substance: 4.606 mg. AgBr (direct precipitation).

$C_{11}H_{16}O_7Br$. Calculated. Br 23.58. Found. Br 24.05

By the action of dry silver acetate upon crystalline bromotriacetyl ribose dissolved in toluene, the original tetracetyl ribose (m.p., 110°) was regenerated. After recrystallization from 95 per cent ethyl alcohol, its rotation was

$$[\alpha]_D^{25} = \frac{-0.90^\circ \times 100}{2 \times 0.828} = -54.3^\circ \text{ (in chloroform)}$$

Action of Methyl Alcohol (in Presence of Silver Carbonate) upon Bromotriacetyl Ribose—30 cc. of dry methyl alcohol were added quickly to an intimate mixture of 2.5 gm. of finely powdered, crystalline bromotriacetyl ribose with 6 gm. of dry, freshly prepared silver carbonate and the mixture vigorously shaken for 30 minutes, after which time no bromine was found in a small filtered test portion. The mixture was filtered, the silver salts well washed with dry ether, and the combined filtrate and washings evaporated to a thick syrup under diminished pressure at room temperature. It was dissolved in dry ether and the small amount of pink, flocculent silver precipitate removed by adding a little charcoal and filtering. The filtrate was allowed to evaporate slowly in a vacuum desiccator to a colorless syrup which crystallized spontaneously. It was recrystallized from a mixture of dry ether and petroleum ether at -15° , being obtained as long rectangular plates, m.p. $77-78^\circ$. Yield (after one recrystallization), 0.495 gm.

Its rotation was

$$[\alpha]_D^{25} = \frac{+0.05^\circ \times 100}{2 \times 1.025} = +2.4^\circ \text{ (in chloroform)}$$

The substance had the following composition.

4.305 mg. substance: 7.845 mg. CO₂ and 2.391 mg. H₂O.

6.200 " " : 4.890 " AgI.

C₁₂H₁₈O₈. Calculated. C 49.64, H 6.3, OMe 10.69

Found. " 49.69, " 6.2, " 10.42

100 mg. substance required 7.33 cc. 0.1 N NaOH (alkaline hydrolysis).

C₈H₁₂O₆ · (CH₃CO)₂. Calculated. COCH₃ 29.7 (for 2 hydrolyzable acetyl groups)

Found. " 31.5

A comparison of the rate of hydrolysis of this substance with those of the γ forms of tetracetyl methylmannoside and triacetyl-methylxyloside⁴ was made. A solution of 100 mg. of substance in 5 cc. of absolute ethyl alcohol was prepared, and this was diluted to 10 cc. with 0.02 N aqueous hydrochloric acid. The rotation was observed immediately. The solution was then heated in a sealed glass tube at 98° for 90 minutes after which the rotation of the cooled solution was again observed. It was strongly reducing towards boiling Fehling's solution.

$$[\alpha]_D^{25} = \frac{+ 0.36^\circ \times 100}{2 \times 1.01} = + 17.8^\circ \text{ (initial)}$$

$$[\alpha]_D^{25} = \frac{- 0.39^\circ \times 100}{2 \times 1.01} = - 19.3^\circ \text{ (final)}$$

Under the same conditions, the *normal* triacetyl methylriboside showed no change in rotation, indicating its relatively greater stability in the presence of 0.01 N hydrochloric acid (alcoholic). The specific rotation both before and after heating in a sealed tube at 98° for 90 minutes was, in this case,

$$[\alpha]_D^{25} = \frac{- 0.37^\circ \times 100}{2 \times 1.445} = - 12.8^\circ$$

The solution was very faintly reducing to boiling Fehling's solution on prolonged boiling.

Preparation of Normal Triacetyl Methylriboside—Polarimetric observations on a 1 per cent solution of crystalline ribose in 1 per cent methyl alcoholic hydrogen chloride at 27° showed that the specific rotation rapidly changed from $[\alpha]_D^{27} = -14.7^\circ$ (2 minutes after admixture) to a maximum value $[\alpha]_D^{27} = -2.4^\circ$ (14 minutes)

and thereafter decreased to an apparently constant value $[\alpha]_D^{27} = -41.5$ (56 hours).

Accordingly, 5 gm. of ribose were dissolved in 50 gm. of cold 1.5 per cent methyl alcoholic hydrogen chloride and kept at room temperature (23°) until the maximum value had been passed (40 minutes). It was boiled gently under a reflux for a further 105 minutes after which it was cooled in ice, shaken with a little charcoal, filtered, and the rotation of the filtrate observed. $[\alpha]_D^{23} = -39.5^\circ$. The hydrogen chloride was now neutralized with silver carbonate, the mixture filtered, and the silver salts extracted several times with boiling methyl alcohol under a reflux. The combined filtrate was evaporated to a thick syrup under diminished pressure at 35°. Slight deposition of silver salt occurred so the syrup was redissolved in methyl alcohol, shaken with a little charcoal, filtered, and again evaporated to a thick syrup (yield, 5.4 gm.). All attempts to obtain either the α or the β form of methylriboside from this mixture were unsuccessful.⁷

5 gm. of syrupy methylriboside were dissolved in a mixture of 20 cc. of acetic anhydride and 25 cc. of pyridine, with cooling in ice. The solution was kept at 0° for 16 hours after which it had assumed a jelly-like consistency. It was poured onto crushed ice with vigorous stirring but, even on standing, only a negligible amount of insoluble gum was deposited. The aqueous liquor was therefore extracted three times with chloroform and the extract washed successively with ice-cold dilute sulfuric acid, ice-cold dilute sodium bicarbonate solution, and ice water until neutral. It was then dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated to a thick syrup under diminished pressure at 35°. All attempts to crystallize the product were of no avail. Yield, 8.5 gm. of syrup.

This was distilled at high vacuum giving a main fraction (weight, 5.2 gm.) boiling at 120° at 0.05 mm. (bath temperature, 140°). This was a very pale yellow, fairly viscous syrup having $n_D^{24} = 1.4523$ and

$$[\alpha]_D^{28} = \frac{-1.00^\circ \times 100}{2 \times 2.879} = -17.4^\circ \text{ (in chloroform)}$$

⁷ Levene, P. A., Jacobs, W. A., and Medigreceanu, F., *J. Biol. Chem.*, **11**, 371 (1912).

Its composition was as follows:

5.991 mg. substance: 10.975 mg. CO_2 and 3.391 mg. H_2O .

5.185 " " : 4.150 " AgI.

$\text{C}_{12}\text{H}_{18}\text{O}_8$. Calculated. C 49.64, H 6.3, OMe 10.69

Found. " 49.95, " 6.3, " 10.57

100 mg. syrupy substance required 10.22 cc. 0.1 N NaOH (alkaline hydrolysis).

$\text{C}_6\text{H}_9\text{O}_6 \cdot (\text{CH}_3\text{CO})_3$. Calculated. COCH_3 44.5 (for 3 hydrolyzable acetyl groups)

Found. " 44.0

A TRIBASIC ACID PRESENT IN LIVER, CONVERTIBLE INTO PYRROLE DERIVATIVES

BY H. D. DAKIN AND RANDOLPH WEST

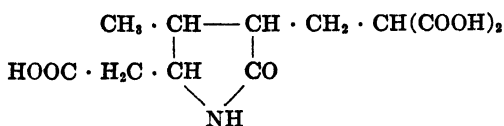
(From the Medical Clinic, Presbyterian Hospital, Columbia University, New York, and Scarborough-on-Hudson, New York)

(Received for publication, April 23, 1931)

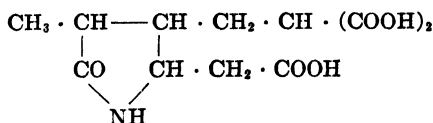
In a previous communication by West and Howe (1), an account was given of a quinine salt of an acid derived from liver, which on hydrolysis gave β -hydroxyglutamic acid. Later, γ -hydroxyproline was identified among the products of hydrolysis and the results indicated that the quinine salt was that of an acid derived from either a peptide or diketopiperazine derivative of the two amino acids just mentioned. Further investigation has confirmed this view as to the chemical nature of the substance. At the same time it was stated that "evidence had been obtained for the presence of a second quinine salt of higher quinine content." The present paper contains a preliminary account of this second salt, and some of the transformations of the acid contained in it. The new quinine salt is sharply differentiated from the one earlier described by West and Howe, by higher melting point, higher optical rotation, lower solubility in water, and the fact that it gives no amino nitrogen on hydrolysis.

In the experimental portion of the paper detailed directions are given for the preparation of the new salt, which may be obtained in considerable yield (up to about 1 per cent), from the powdered liver extract employed. The acid is tribasic, and on elementary analysis gives results indicating that it is represented by the formula $C_{11}H_{15}O_7N + H_2O$. For reasons which immediately follow we tentatively suggest that the structure of the acid may be represented by one of the two following formulæ, which it will be noted only differ in the relative positions of the α and α' substituents. Some reasons exist which lead us to regard Formula I as preferable to Formula II, and for the sake of simplicity it will be adopted with reservation in the subsequent discussion.

Tribasic Acid of Liver



I



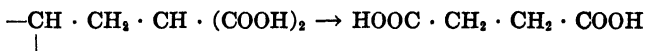
II

The presence of three carboxyl groups is shown by direct titration with caustic alkali with phenolphthalein as indicator. Addition of either alcohol or neutralized formaldehyde to the neutralized solution is not followed by the slightest change in the neutral point indicating the absence of carboxyl groups adjacent to a carbon atom carrying an amino or imino group. The presence of three carboxyl groups is also indicated by the fact that the neutral quinine salt is composed of 2 molecules of the acid with 3 of the quinine, thus resembling citric and analogous tribasic acids.

No free amino group is shown by reaction with nitrous acid either before or after treatment with boiling hydrochloric acid. The free acid does not immediately reduce potassium permanganate in the cold in either sodium carbonate or dilute sulfuric acid solution and hence does not contain ordinary unsaturated carbon linkages. The diazo reaction is completely negative.

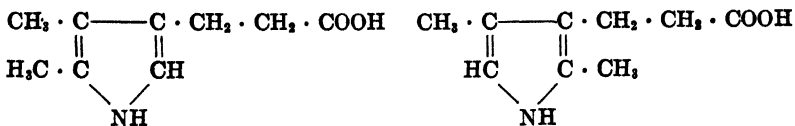
The acid gives a chocolate-colored periodide compound with free iodine in acid solution but this is not due to the presence of alkyl groups attached to nitrogen. Hydriodic acid yields no alkyl iodide and when heated with barium hydroxide under pressure, the portion of nitrogen split off from the main molecule is found to be simply ammonia. The fate of the rest of the nitrogen will be referred to below. Both the acid and its salts are strongly levorotatory and it will be noted that the proposed structure includes 3 asymmetric carbon atoms. The evidence for the presence of two adjacent carboxyl groups is based on the fact that the barium salt, on being warmed in aqueous solution to the moderate temperature of about 80° in a closed tube, yields

a molecular proportion of barium carbonate in the course of a few minutes. With the known absence of a β -ketonic acid grouping, this result can hardly be interpreted otherwise than by the assumption that the acid is a substituted malonic acid derivative. Evidence for the presence of the methylmalonic acid side chain is found in the fact that oxidation of the acid with nitric acid gives a good yield of succinic acid, the formation of which is clearly to be expected.



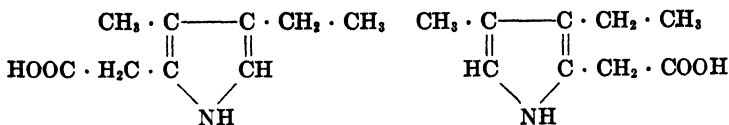
It has already been stated that the acid readily parts with a molecule of carbon dioxide when its barium salt is warmed in aqueous solution. If excess of barium hydroxide is added and the heating continued in a closed tube to a higher temperature, *e.g.* 120–130°, a series of reactions occurs possessing considerable interest. In the first place, a second molecule of carbon dioxide is removed and a small amount of ammonia is liberated. But most of the nitrogen is now to be found in the form of an ether-soluble monobasic pyrrolecarboxylic acid. The acid has the formula $\text{C}_9\text{H}_{13}\text{O}_2\text{N}$ and is readily isolated in the form of a sparingly soluble picrate. It forms a silver salt containing 1 equivalent of metal, reduces permanganate instantly, gives all the typical reactions of a pyrrole derivative including the pine splinter reaction, coupling with diazo salts in acetic acid solution, and an intense color reaction with dimethylaminobenzaldehyde, also a strong iodoform reaction. It will be noted that the acid has the same empirical formula as the hemopyrrolecarboxylic (III) and cryptopyrrolecarboxylic acids (IV) obtained by Piloty (2) and by Fischer and Röse (3) from blood pigment. Since the melting point of the picrate is considerably higher (182°) than that of the picrate of hemopyrrolecarboxylic acid (148°) and of cryptopyrrolecarboxylic acid (153°), the alternative Formulæ V or VI may be provisionally assigned to the acid with some degree of plausibility. The complete absence of hemo- and cryptopyrrolecarboxylic acids is not certainly established, however.

The formation of an unsaturated pyrrole acid by the action of heat on a saturated acid appears to present considerable novelty and it is hard to find satisfactory analogies for the reaction,



III

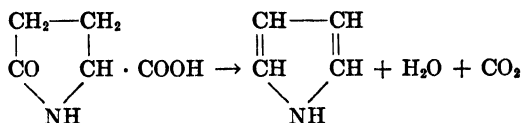
IV



V

VI

although the formation of pyrrole by the distillation of pyrrolidone-carboxylic acid presents some points of similarity.¹



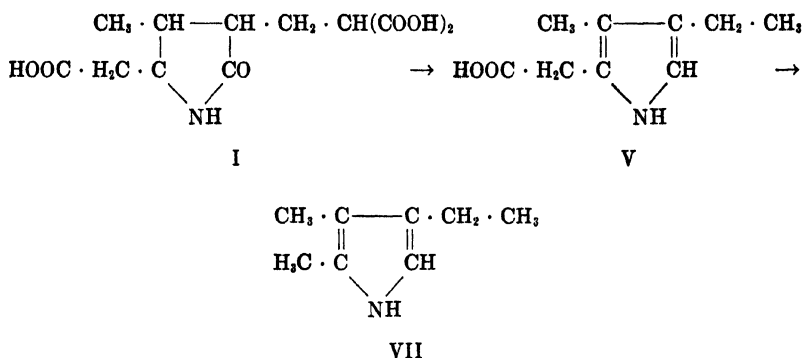
Tafel and Wassmuth (4), moreover, observed the formation of pyrrole on melting sodium pyrrolidone. It will be noted that the formation of the pyrrole acid as represented involves the elimination of a molecule of water, as well as of carbon dioxide, and a change in the position of hydrogen atoms by intramolecular rearrangement. In conformity with this conception, it is noted that the optical activity of the parent acid completely disappears in the course of the reaction, the loss of asymmetry being a necessary consequence of the change in position of the 3 hydrogen atoms involved.

If the conditions of heating the barium salt of the parent acid are changed so that the temperature is maintained at 155–165°

¹ In order to dry and to obtain a better analogy the neutral barium salt of pyrrolidonecarboxylic acid was heated with water in sealed tubes. At temperatures up to 165° no evidence of pyrrole formation could be demonstrated but at 180° small amounts of a pyrrole compound, presumably pyrrole- α -carboxylic acid could be identified. Naturally the conditions of these experiments differed somewhat from the preceding ones since no excess of barium hydroxide could be used, as alkali would at once open the pyrrolidone ring and give glutamic acid.

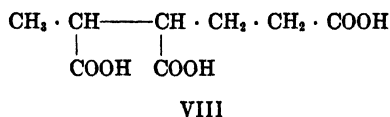
for 5 or 6 hours in the presence of excess of barium hydroxide, a further reaction occurs and none of the pyrrolecarboxylic acid is to be found in the solution. Instead, a volatile pyrrole base, extremely sensitive to oxidation, is obtained. It gives all of the typical reactions associated with hemopyrrole (VII), including an unstable picrate melting at 110° , which gave fairly satisfactory results on analysis. The yield of pyrrole base was small and no opinion can be offered as to whether it was a mixture of isomers such as is found in hemopyrroles derived from hemin.

The formation of the pyrrole acid and pyrrole base from the parent acid may be represented tentatively as follows:



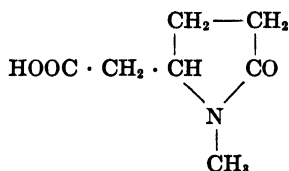
It is noteworthy that the sealed tubes in which the preceding experiments were carried out showed no pressure on opening and remarkably little pigmentation of the contents occurred, indicating a fairly smooth course of reaction.

It will be noted that the structure of the parent acid as provisionally assigned represents it as closely related to pentane- β - γ - ϵ -tricarboxylic acid (VIII), an acid synthesized by Haworth and Perkin (5) and later obtained by Kuster (6) in two isomer forms by the reduction of his tribasic hematinic acid derived from hemin.



Preliminary experiments to obtain pentane- β - γ - ϵ -tricarboxylic acid from the liver acid by oxidation have given promising but not entirely conclusive results. The experiments will be continued when material permits. On oxidation with chromic acid in sulfuric acid solution under conditions similar to those used by Willstätter (7) for the oxidation of tropinic and ecgoninic acids to methyl succinimide, both substances being structurally related to the acid from liver, it was easy to establish the formation of a succinimide derivative but its amount was not sufficient for satisfactory identification. The experiments at least furnish strong presumptive evidence of the presence of a ring containing 4 carbon atoms and 1 nitrogen atom.

In considering possible objections to the structure assigned to the new acid the most obvious criticism might be based on the fact that the formula contains a pyrrolidone ring and yet on boiling the substance with mineral acid no opening of the ring occurs with formation of an amino and carboxyl group, as in the case of pyrrolidonecarboxylic acid. This objection is however illusory, for several pyrrolidone derivatives are known, in which the ring is not opened by hydrolysis, and one of these, ecgoninic acid (IX), presents a perfect analogy to the case in point, as will be seen by a comparison of the respective formulæ.



IX

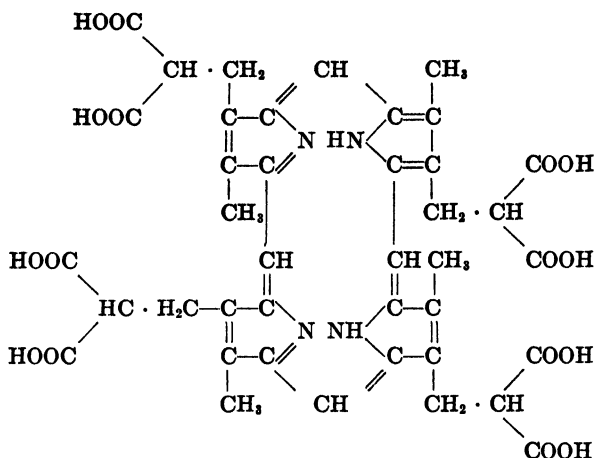
Ecgoninic acid was shown by Willstätter and Hollander (8) to be entirely resistant to hydrolysis by acids at 120° , by strong alkali, and by silver oxide, and moreover the methylaminoadipic acid which it might be expected to yield apparently has no separate permanent existence and when formed by synthesis at once passes over into the pyrrolidone derivative. The striking similarity between the behavior of the acid from liver and ecgoninic acid furnishes some reason for assigning the third carboxyl to the position given rather than attached to the β -methyl group.

The formation *in vitro* of pyrrole derivatives from the tribasic acid from liver raises the question as to whether these observations have any biochemical significance. We are inclined to the view that the answer is in the affirmative. It will be recalled that the acid is separated from a mixture of substances precipitable by phosphotungstic acid containing material some of which is highly potent in producing an increase in reticulocytes (presumably as the result of hemoglobin synthesis) when administered, under suitable conditions, to cases of pernicious anemia. When the new acid is administered in the form of a single dose (0.25 to 0.75 gm.) intravenously, care being taken to avoid loss of carbon dioxide from the acid by undue heating or otherwise, we have found that in some cases a distinct rise in reticulocytes has followed, but the rise is neither as prompt nor as quantitatively striking as that produced by structurally related substances. We hope to describe these substances in greater detail in a later communication. In any case we wish it clearly understood that we do not regard the new acid as responsible for any large share in the reticulocyte response evoked by liver extract.

The reason for the relatively lower activity of the tribasic acid as a possible precursor of hemoglobin would appear easily understood on consideration of the structural formulæ of the various porphyrins, which are mainly due to the work of Fischer (9). The formula which Fischer gives for uroporphyrin is given below; while that of coproporphyrin results from the replacement of four carboxyl groups by hydrogen.

It will be noticed at once that the characteristic methylmalonic acid side chain present in one acid occurs in each of the four pyrrole rings of uroporphyrin and that simply from the chemical standpoint our acid would appear more directly allied to uroporphyrin than to coproporphyrin or hematoporphyrin for the synthesis of the latter substances would require the elimination of 4 and 6 molecules of carbon dioxide respectively from each group of four pyrrole rings in the porphyrin nucleus.

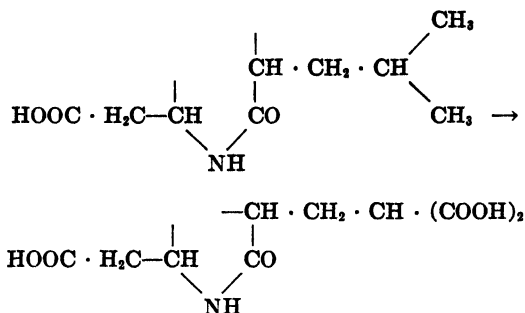
The investigations of Keilin and of Fischer and Hilmer, have shown that coproporphyrin is formed in significant amount by yeast grown in pure culture, especially in vitamin-poor media. The possible relationship of our substance to this biochemical synthesis invites speculation, especially as to the synthetic



materials, of necessity simple in character, which might lead to the production of the new acid. The possibility of the methylmalonic acid side chain being evolved as the result of the oxidation of some substance containing the leucine skeleton such as, for example, leucyl- β -alanine is sufficiently obvious to invite further research.

EXPERIMENTAL

The following method for the preparation of the new acid, in the form of its quinine salt, differs from that used by West and Howe (1) mainly in the use of basic lead acetate in the earliest stage of the process instead of later and largely gets rid of the peptide acids. 300 gm. of liver extract powder (Eli Lilly and Company) are stirred with 500 cc. of water and without filtering basic lead acetate (100 gm.) in 200 cc. of warm water is added. The mixture is filtered on a fluted filter paper and the precipitate rejected. Washing of the precipitate is hardly advisable, especially as it is desirable to keep the volume of fluid low. Sulfuric acid (1:2) is added to the filtrate in such amount that practically the whole of the lead is precipitated but free sulfuric acid is absent as shown by the failure to blue Congo paper—about 45 cc. are usually required. Without the filtering off of the lead sulfate, 50 gm. of finely powdered picric acid are added, the whole shaken well and allowed to stand for a few hours, after which the pre-



cipitate is filtered off with suction, washed, and rejected. The clear filtrate is then extracted in a separatory funnel with a mixture of equal parts of either butyl or amyl alcohol and ether, 150 cc. of each solvent being used. After five such extractions the aqueous layer is rendered acid to Congo paper by the addition of a small quantity of 1:2 sulfuric acid—usually about 10 cc.—and the bulk of the residual picric acid is then removed by repeating the extraction with ether. The object of the preceding operation is to effect the removal of a variety of mono- and diamino acids and other bases.²

A current of air is blown through the aqueous layer remaining from the preceding extractions in order to remove some of the residual dissolved ether, although its complete removal is not necessary. Sulfuric acid is then added to bring the concentration up to about 2.5 per cent and phosphotungstic acid added till precipitation is complete. The precipitate is at first finely divided and later usually coalesces to form somewhat sticky lumps. The precipitate is superficially washed with dilute sulfuric acid and the filtrate rejected.

The decomposition of the phosphotungstate precipitate requires considerable care or the whole of the acid sought may easily be destroyed. The following method has given good results but can

² The use of immiscible solvents for the extraction of amino acids and bases as picrates, even when no obvious formation of a sparingly soluble picrate can be observed, offers some degree of novelty and may be of use in a variety of ways. It was found that when approximately decinormal solutions of various amino acids were mixed with excess of picric acid and then extracted with an equal volume of amyl alcohol and ether, much of the amino acid passed into the solvent layer and could be recovered on shaking

probably be improved upon.³ The phosphotungstate precipitate is dissolved in about 200 cc. of warm acetone in a large beaker, which is then cooled to room temperature. Small successive quantities of saturated warm barium hydroxide solution are then added with vigorous stirring, a significant rise in temperature being avoided by cooling the contents of the beaker after each addition of barium hydroxide. It is most important to avoid local excess of hot barium hydroxide and with this end in view it is well to filter the mixture as soon as a slight alkaline reaction to litmus has been established, even if phosphotungstic acid is still present in the filtrate. The filtrate is reserved and decomposition of the precipitate completed in the usual way. The second filtrate is combined with the first and any necessary addition of barium hydroxide made in order to complete the removal of phosphotungstic acid. In the event of excess of barium hydroxide being present at any stage its removal is cared for by the prompt passage of a current of carbon dioxide gas. Dilute sulfuric acid is then added to the filtrate in such amount that part but not all of the barium present is precipitated. The reaction of the liquid should be definitely acid to litmus but must not turn Congo paper blue. The solution is then filtered from barium sulfate and concentrated

the latter with dilute sulfuric acid. The solvent layer containing most of the picric acid, after being washed to remove traces of sulfuric acid may be again used for extraction. A few roughly qualitative experiments are indicated in which the volume of amino acid solution (25 cc.), saturated with picric acid, was equal to the solvent volume and in which three extractions were made by simple shaking in a separatory funnel. The hexone bases, histidine, arginine, and lysine, were extracted to the extent of 88 to 90 per cent, leucine was very readily extracted, 90 per cent being in the first extract; valine and phenylalanine behaved similarly. Choline was extracted to the extent of 50 per cent, while only 12 per cent of betaine was recovered. Glutamic acid was extracted to the extent of 80 per cent, while under similar conditions only 10 to 14 per cent of pyrrolidonecarboxylic acid was recovered. Histamine and adrenalin are easily and almost quantitatively extracted under similar conditions from very dilute solutions.

³ More recently we have found that adding the acetone solution from a dropping funnel to a vigorously, mechanically stirred suspension of freshly slaked lime has some advantages. The whole operation is conducted at room temperature and after filtration the calcium is removed as oxalate.

to a thin syrup by evaporation under reduced pressure at a temperature not exceeding 45°. Precipitation of the acids as barium salts is next effected as follows: A little alcohol, insufficient to produce a permanent precipitate, is added to the residue in the flask, which is then cooled in a bath of water. Barium hydroxide is dissolved by boiling with its own weight or less of water and this is added by degrees to the mixture in the flask, being cooled after each addition. The addition of barium hydroxide is continued until the mixture is definitely alkaline to phenolphthalein, and then about a liter of alcohol is added by degrees with vigorous shaking. The precipitated barium salts are filtered off with suction, washed with alcohol, and suspended in water. Decomposition is effected by the cautious addition of sulfuric acid until a slight positive reaction towards Congo paper is observed and the barium sulfate removed by filtration. The filtrate is examined for either excess of barium or sulfuric acid, and treated accordingly. It is preferable to allow a trace of barium to remain in the solution rather than have any sulfuric acid present since the latter if present is apt to reappear as basic quinine sulfate and cause considerable confusion. The solution is then concentrated under reduced pressure to a volume of about 100 cc., and then exactly neutralized to litmus by the addition of quinine dissolved in alcohol—usually 4 to 6 gm. are required. A small amount of sticky pigmented material is apt to separate at this stage and is readily removed by filtration. The clear filtrate in a crystallizing dish is allowed to evaporate slowly in a warm place and is placed in the refrigerator as soon as crystals make their appearance. A second crop may usually be obtained by concentrating the mother liquor. The crystalline salt is sucked off on a small funnel and washed with a little cold water and then transferred to a porous plate. The yield of crude salt varies considerably, depending both on the particular liver preparation and the success with which decomposition of the unstable acid has been avoided. In good experiments the yield of well purified quinine salts has reached 1 per cent of the liver extract employed. Usually when the yield has been disappointing, a correspondingly larger amount is obtained of the non-crystallizable quinine salt of the acid formed from the former by loss of carbon dioxide.

The quinine salt is recrystallized best from 30 per cent alcohol

with about 12 cc. for each gm. of crude product, any prolonged heating with the solvent being avoided. The crystals separate in the form of fine prismatic needles melting sharply at 149–150°, and are not very easily soluble in water or dilute alcohol when thoroughly purified. The following analyses refer to different preparations, dried at not over 60°.

Analysis

0.1172 gm. substance:	0.2711 CO ₂ and 0.0728 H ₂ O.	
3.525 mg. " :	8.170 " " 2.16 " .	
3.560 " " :	8.22 " " 2.18 " "	
3.695 " " :	0.239 N at 28° and 784 mm.	
0.1205 gm. " :	0.0104 NH ₃ (Kjeldahl).	
Calculated for C ₁₁ H ₁₅ NO ₇ ·H ₂ O·(C ₂₀ H ₂₄ N ₂ O ₂) _{1.5} .	C 63.3, H 6.82, N 7.21	
	" 63.1, " 6.90	
	" 63.2, " 6.86, " 7.22	
	" 63.0, " 6.83, " 7.17	

The specific rotation was determined in 25 per cent alcoholic solution.

$$c = 1.0 \quad l = 2.2 \quad \alpha = 3.23^\circ$$

$$[\alpha]_D^{20} = -147^\circ$$

The amount of quinine in the salt was determined by decomposing the salt with sodium hydroxide and extracting the base with chloroform. The following results were obtained: 62.7, 62.9, 64.5 compared with a calculated value of 62.5. The free acid is obtained by decomposing the quinine salt with a slight excess of barium hydroxide of known concentration, removing the quinine by chloroform or ether extraction, and then removing the barium by the addition of an exact equivalent of sulfuric acid. On concentrating at a low temperature in a desiccator, a syrup is obtained which does not readily crystallize but sets finally to a glassy mass showing some signs of crystalline structure on fracture.

The ammonium salt crystallizes well in needles which are only moderately soluble in water. Addition of silver nitrate to the neutral ammonium salt in dilute solution precipitates very little silver salt even on addition of alcohol. The copper salt was obtained by digesting a solution of the acid with freshly precipi-

tated copper hydroxide and concentrating slowly below 60°. On filtering into alcohol, a fine apple-green copper salt is obtained which becomes still lighter on drying. The titration equivalent of the acid is better determined on the crystalline quinine salt than on the imperfectly crystalline acid. A known weight of salt was decomposed with a known excess of decinormal sodium hydroxide, the quinine removed by extraction with freshly distilled chloroform of tested neutrality, and then the excess of alkali determined by titration with phenolphthalein as indicator.

0.0510 gm. acid neutralized	5.25 cc. 0.1 N sodium hydroxide.
0.1000 " " " "	10.2 " 0.1 " " "

The values calculated on the basis of a tribasic acid, $C_{11}H_{15}NO_7 \cdot H_2O$, are 5.26 cc. and 10.3 respectively. The titration value was not changed by the addition of alcohol or of neutralized formaldehyde.

The main properties of the acid have already been given in the introduction to the discussion of the structure of the acid and need not be repeated. It is noteworthy that the acid gives only a feeble pyrrole pine splinter reaction when simply distilled with soda lime or solid baryta, a fact that is somewhat surprising in view of its yielding pyrrole derivatives under other conditions. It may be recalled, however, that Angeli and Piéroni (10), have shown that pyrrolidone itself under similar conditions gives no pyrrole. The acid gives a fluorescent green color on warming with 70 per cent sulfuric acid and a trace of solid β -naphthol, similar to that given by malic and β -hydroxyglutamic acids. The absence of amino groups reacting with nitrous acid was determined on both the original salt and after boiling with 20 per cent hydrochloric acid for an hour and subsequently removing the excess of acid by evaporation. 50 mg. portions gave no more than 0.1 cc. of nitrogen gas after 5 minutes reaction time, whereas, had an amino group been present, over 3 cc. would have been obtained. On standing for several hours, a trifling increase in the gas volume was noted.

It was of special importance to prove the absence of any alkyl group attached to nitrogen especially in view of the difficulty encountered years ago in detecting the methylamino group in the analogously constituted ecgoninic acid. The acid (0.5 gm.) was

therefore heated in a sealed tube with barium hydroxide (2 gm.) and water, 10 cc. for 5 hours at 140° . The contents of the tube were distilled into excess of hydrochloric acid and evaporated to dryness. A small amount of volatile pyrrole was destroyed by evaporating with the acid and the residual hydrochloride was insoluble in alcohol. Its amount was equivalent only to about 10 per cent of the total nitrogen. It was converted into the platinum salt, which had no melting point and analyzed with the following results: Pt 43.8, H 2.06, N 6.30 per cent. Calculated for $(\text{NH}_4)_2\text{PtCl}_6$: Pt 43.8, H 1.80, N 6.36. Only a questionable trace of carbon could be detected on analysis. The results clearly indicate that the salt was practically pure platinum ammonium chloride and hence that no alkyl group is attached to nitrogen in the parent acid. It will be noted that in the suggested formula for the acid the arrangement is such that the opening of the pyrrolidone ring would result in the formation of a β -alanine derivative, and the formation of ammonia from such a grouping under the influence of alkali at high temperature might reasonably be expected. It should be understood that liberation of ammonia from the acid under the influence of alkali only takes place at high temperatures under pressure. No ammonia is liberated on a simple distillation in dilute solution with excess of sodium hydroxide.

The action of barium hydroxide on the acid was investigated under three types of conditions. The solutions were prepared by decomposing the crystalline quinine salt with excess of barium hydroxide, removing the quinine by chloroform extraction, and then heating in a sealed tube. In the experiments at temperatures above 100° the glass tube was placed in a welded steel tube containing water and closed with the help of a leaden washer and threaded screw. The whole arrangement was then heated in a paraffin bath to the required temperature. A sufficient number of observations has not been made to determine the best experimental conditions but the following experiments in which approximately 1 gm. of the barium salt was heated with 2 gm. of barium hydroxide and 10 cc. of water may be taken as typical.

As stated in the introduction, carbon dioxide is removed from the barium salt with great ease. On warming the clear solution

in a water bath, the separation of barium carbonate is noticeable at about 70° and proceeds rapidly until after short heating in a boiling water bath barium carbonate equivalent to 1 molecule of carbon dioxide is quickly precipitated. At this temperature no indication of any pyrrole derivative has been observed. On carrying the heating further at 125–140° for 5 hours, more barium carbonate is found and in addition a pyrrole acid makes its appearance, together with trivial amounts of a volatile pyrrole. The picrate and silver salt of the pyrrole acid were characterized as follows: The alkaline contents of the tube were filtered and extracted a couple of times with ether and then made just acid to Congo red with sulfuric acid and again filtered. The pyrrole acid is now extracted with ether. The ether residue which is mainly crystalline shows all the typical reactions of a pyrrole compound, *e.g.* dimethylaminobenzaldehyde reaction, coupling with diazo salts in acetic acid solution, pine splinter reaction, iodoform reaction, and immediate reduction of potassium permanganate. The acid was neutralized with ammonia and then precipitated with silver nitrate. The silver salt is a curdy, sparingly soluble precipitate retaining all the above pyrrole reactions. It was dried *in vacuo* in the dark and analyzed as follows:

Analysis

4.900 mg. substance: 1.960 mg. Ag.

5.215 “ “ : 7.475 “ CO₂ and 1.970 mg. H₂O.

7.360 “ “ : 0.340 cc. N at 25° and 760 mm.

C₉H₁₁NO₂Ag. Calculated. C 39.4, H 4.38, N 5.11, Ag 39.4

Found. “ 39.1, “ 4.19, “ 5.13, “ 40.0

The picrate of the acid is most conveniently obtained without previous extraction of the acid by ether. The alkaline solution is first extracted with ether, the residual aqueous solution then made acid with sulfuric acid and filtered, and then a slight excess of sodium acetate added so that the solution is no longer acid to Congo red. On addition of cold saturated aqueous picric acid, the picrate separates in the form of clumps of microscopic needles. The picrate is sparingly soluble in ether and in ethyl acetate, and is best crystallized from 75 per cent alcohol. It melts at about 182° with slight previous softening.

*Analysis*5.25 mg. substance: 8.97 mg. CO₂ and 1.92 mg. H₂O.

3.794 " " : 0.483 cc. N at 24.5° and 756 mm.

C₈H₁₃NO₂·C₆H₅(OH)(NO₂)₃. Calculated. C 45.5, H 4.04, N 14.2
 Found. " 46.5, " 4.06, " 14.5

If the reaction between the acid and excess of aqueous barium hydroxide is carried out at about 160° for 5 or 6 hours, the pyrrole acid just described is no longer to be found in the solution. Under these conditions, the third carboxyl group of the parent acid is removed. The reaction mixture was subjected to a short steam distillation, the distillate saturated with ammonium sulfate, and extracted with ether. On evaporation of the ether a small amount of oil was obtained which rapidly turned brown on exposure to air. It showed all the reactions of hemopyrrole and gave an insoluble compound with mercuric chloride as described by Nencki. The picrate was prepared by adding a saturated ether solution of picric acid to the concentrated ether solution of the base, when it at once separated. It was washed with ether and analyzed. The melting point was 110–112°, with complete decomposition occurring around 125°. The picrate readily darkens on exposure or on heating with solvents.

*Analysis*5.140 mg. substance: 9.125 mg. CO₂ and 2.20 mg. H₂O.

3.17 " " : 0.45 cc. N at 25° and 760 mm.

C₈H₁₃N·C₆H₅(OH)(NO₂)₃. Calculated. C 47.8, H 4.55, N 15.9
 Found. " 48.4, " 4.75, " 15.7

The oxidation of the new acid with production of succinic acid was effected as follows: 1 gm. of the barium salt was dissolved in 10 cc. of water and 10 cc. of nitric acid (sp. gr. 1.42) were added and the whole heated on the water bath, in a covered dish. A vigorous reaction ensued with evolution of much gas. After an hour the cover was removed and the mixture evaporated on the steam bath, further concentrated nitric acid (10 cc.) was then added, and the evaporation repeated. Finally most of the nitric acid was removed by adding water and again evaporating. The syrupy residue deposited large crystals of succinic acid which could easily be separated mechanically from other oxidation products. The latter were insoluble in alcohol and ether and hence the succinic acid could easily be recovered by ether extraction. The acid

was recrystallized from dilute hydrochloric acid and melted at 182°. The yield of succinic acid was considerable. Other oxidation products were acetic and oxalic acids and an acid containing nitrogen soluble in water but sparingly soluble in alcohol and ether, which has not been identified.

Analysis

0.0873 gm. substance: 0.1280 CO₂ and 0.0388 H₂O.

C₄H₆O₂. Calculated. C 40.7, H 5.08

Found. " 40.0, " 4.95

Our grateful thanks are due to Doctor Hans T. Clarke and colleagues for affording us various laboratory facilities of his Department.

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THE ANALYSIS OF WHOLE BLOOD

I. THE PRECIPITATION OF THE PROTEINS .

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Numerous recent investigations have demonstrated that the Folin-Wu tungstic acid method for the precipitation of blood proteins is not altogether a satisfactory preliminary step for the analysis of whole blood. Studies reported by Van Slyke, Folin, Somogyi, Herbert and collaborators, and others have shown that tungstic acid permits non-fermentable reducing materials to pass into filtrates from whole blood, so that accurate determinations of fermentable sugar on tungstic acid filtrates by means of the older sugar reagents are possible only through the use of a complicated procedure involving determinations before and after fermentation with washed yeast. At least one non-protein compound (thioneine), present in whole blood, may be largely precipitated by tungstic acid along with the blood proteins.

In a recent communication from this laboratory (1), the use of tungstomolybdic acid was suggested as a precipitant for blood proteins and it was pointed out that this new precipitant yields filtrates from whole blood containing all of the organic non-protein constituents now known to exist in blood. It was stated that further studies on the determination of certain blood constituents in this filtrate would be reported. It is the purpose of the present paper to revise slightly the directions for preparation of the tungstomolybdic acid precipitating reagent, and as preliminary to the two papers which follow, to discuss some of the questions which have recently arisen in connection with blood analysis.

The revised directions for the preparation and use of the tungstomolybdic precipitating reagents are as follows: 10 gm. of

pure, ammonia-free molybdic acid¹ are treated in a flask with 50 cc. of *N* sodium hydroxide solution and the mixture is boiled gently for from 4 to 5 minutes. The mixture is then filtered, the filter being washed with about 150 cc. of hot water. The total filtrate is cooled and mixed with a solution of 80 gm. of sodium tungstate dissolved in about 600 cc. of water. This mixed solution is diluted to 1 liter. The acid employed during the precipitation is 0.62 *N* sulfuric acid, prepared by diluting 620 cc. of *N* acid to 1 liter. The precipitation of the blood proteins is carried out exactly as in the familiar tungstic acid precipitation. Whole blood is diluted with 7 volumes of water, 1 volume of the mixed tungstomolybdate solution is added, followed by 1 volume of the 0.62 *N* sulfuric acid. For precipitation of plasma 8 volumes of water, followed by 0.5 volume of the tungstomolybdic acid and of the 0.62 *N* sulfuric acid, are used, and for precipitation of corpuscles alone, 5 volumes of water and 2 volumes of each of these solutions are employed.

In the two following papers of this series (2) it is shown that sugar and uric acid can be determined simply and with accuracy upon tungstomolybdic acid filtrates from whole blood. In the previous paper dealing with tungstomolybdic acid precipitation, it was shown that the other non-protein constituents of blood may be determined upon these filtrates. Hence we are now prepared to recommend the use of tungstomolybdic acid as a protein precipitant preliminary to the analysis of whole blood, plasma, or corpuscles. Unlike the other methods of precipitation now available, the new precipitant yields filtrates upon which any one or all of the non-protein constituents of blood may be accurately determined.

In view of the special methods of precipitation recently advocated by some investigators (Somogyi, Folin, Herbert and Bourne), it seems desirable to discuss briefly whether it is necessary or advantageous to adopt methods which limit the number of constituents which may be determined upon a blood filtrate, and

¹ The molybdic acid used should be of the best grade, labeled to contain no ammonia or a "trace." The Eimer and Amend "tested purity, special" is very satisfactory. The ammonia content of the high grade molybdic acids is, according to our determinations, less than that of most of the high grade sodium tungstate samples.

which may yield figures representing the composition of neither whole blood nor plasma.

Somogyi (3) has suggested the use of zinc, copper, or iron salts as precipitating agents for blood proteins where sugar determinations are to be made. In the following paper of this series we shall discuss one of Somogyi's procedures in some detail.

Folin (4) and Herbert and Bourne (5) have independently and almost simultaneously suggested what constitutes a real innovation in blood analysis. These investigators have suggested that blood be precipitated without laking the corpuscles. Herbert and Bourne developed their suggestion for analysis of unlaked blood as the result of their conclusion that glutathione is present only in the corpuscles and is the only compound existing in blood in appreciable amounts which is responsible for the non-fermentable reducing fraction. Thus Herbert and Bourne stressed the use of unlaked blood for analysis only in connection with the determination of sugar.

Folin saw new possibilities in the use of unlaked blood for analysis beyond the apparent elimination of the non-sugar reducing fraction, and has strongly advocated adopting analysis of filtrates from unlaked blood in place of filtrates from whole blood. The material in blood (undoubtedly chiefly thioneine) which causes the high figures for uric acid by the direct methods is chiefly (or wholly) confined to the corpuscles. Hence Folin concluded that more accurate figures for uric acid as well as for sugar can be obtained from unlaked blood filtrates. A third consideration which has appealed strongly to Folin is the fact that where unlaked blood filtrates are employed the fraction of "undetermined" nitrogen disappears. In this connection Folin and Svedberg (6) say, "the conclusion seems inescapable that for nearly every kind of metabolism study, it must be obscuring and misleading to include in the analyses of blood as a transportation system, an unknown mixture of residues obtained from the destroyed cellular elements which have no direct connection with the circulating waste products or food products."

We frankly admit that there is much appeal in the arguments which Folin has advanced in favor of his proposed innovation in blood analysis. Yet we believe that careful consideration will lead to the conclusion that the new technique does not offer

sufficient advantages to offset the limitations which necessarily go with it.

Is there, for instance, sufficient warrant for Folin's assertion that the contents of the red blood cells "have no direct connection with the circulating waste products or food products"? We believe that the data available concerning the constituents of the red blood cells are by no means sufficient to warrant any such conclusion. The mere fact that a substance is found largely confined to the corpuscles under the conditions obtaining in blood *in vitro*, is not proof that this substance does not contribute to tissue metabolism. It would seem that too little is known of the significance of the glutathione, thioneine, and other constituents of the corpuscles to warrant a definite conclusion with regard to their functions. We have already reported from this laboratory a marked increase in thioneine in the blood in some cases of diabetes (7). Obviously this question and the question of the real rôle of glutathione and of other constituents of the corpuscles deserve further study. Unless absolutely necessary, it seems undesirable to halt progress in the study of these interesting questions by arbitrarily eliminating these compounds from the field of investigation.

In the two following papers we shall show that determinations of sugar and of uric acid may be carried out very simply and with accuracy upon filtrates from whole blood, and we shall show that for a correct picture of the true and the non-fermentable "sugar" in either whole blood or plasma, it is necessary to employ a reagent specifically constructed to meet the conditions obtaining in blood analysis. We shall also describe a simple procedure for determination of the unfermentable reducing fraction of whole blood. In view of the widely differing figures for this fraction in different bloods, it appears that the question deserves study. Such work cannot be carried out upon "incomplete" filtrates, but with whole blood filtrates one may, at his discretion, determine any one or all of the constituents present in blood.

Contrary to the view expressed by Folin, we believe that the "undetermined" nitrogen in blood does not lead to misinterpretations of clinical or purely investigative findings, and we feel that its presence constitutes nothing less desirable than an interesting and fertile field for investigation.

If figures for whole blood or for plasma are desired, we feel that whole blood or plasma should be analyzed, rather than a fluid (unlaked blood) which is neither whole blood nor plasma, and nearly one-half of the volume of which is made up of shrunken red blood cells containing some apparently non-diffusible compounds (glutathione, thioneine), some partially diffusible compounds (uric acid, amino acids), and some freely diffusible compounds (sugar, urea).

It would seem that in any investigation of the blood, whether clinical or experimental, a complete picture of the composition of the blood should be possible. Since, with methods now made available, sugar and uric acid may be accurately and simply determined upon filtrates from whole blood, we feel that there remains not even a valid theoretical reason for altering the current basic concepts of analysis of complete blood filtrates, which have led to rapid and continued progress in this field. It would seem that advancing knowledge calls simply for certain refinements of analytical technique rather than overthrow of the basic principles of blood analysis.

SUMMARY

A slightly modified technique is described for precipitation of blood proteins with tungstomolybdic acid.

The question of the adoption of unlaked blood as a basis for blood analysis is discussed, and it is concluded that general adoption of such a procedure is not necessary or desirable.

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THE ANALYSIS OF WHOLE BLOOD

II. THE DETERMINATION OF SUGAR AND OF SACCHAROIDS (NON-FERMENTABLE COPPER-REDUCING SUBSTANCES)

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INTRODUCTION

If the established practice of the analysis of whole blood filtrates is to be continued, it is highly desirable that a specific reagent for sugar determination be available for the direct analysis of such filtrates. The error involved through the use of the older sugar reagents (Lewis-Benedict, Folin-Wu) is too great to be permissible although either of these methods appears to furnish figures upon which it is perfectly safe to base clinical interpretations. The reagent proposed in 1928 by the present writer (1) permits accurate determination of sugar directly in filtrates from most bloods, but has objections which limit its general usefulness. The reagent is rather complicated in formula. Probably one factor which has militated against its use has been lack of confidence in it because of the fact that while it appears to yield accurate results for blood sugar, the solution shows considerable reduction when heated with fermented blood filtrates. This fact gives to the reagent something of the nature of a "trick" solution. Although Somogyi and Herbert and Bourne have fully substantiated the writer's contention that the reagent yields essentially correct sugar values for tungstic acid filtrates, we have felt that the solution could not be considered altogether satisfactory, since with bloods low in sugar content the effects of the non-fermentable reducing substances are almost certain to become apparent. We have had in mind the development of a highly specific copper

reagent which could be kept in one solution for at least reasonable periods of time without deterioration, and which should be available for use in a micro form as well as for the ordinary macro determination, so that the advantages of a specific sugar reagent might be extended to determinations made upon minute quantities of blood.

The new copper reagent to be described below meets all of the requirements which we originally had in mind and also provides, by means of simple comparative determinations, a ready method of determining the non-fermentable copper-reducing fraction present in blood. Before proceeding with a description of the new reagent and methods we wish to discuss briefly some general aspects of the question of the non-fermentable reducing materials in blood.

First, we venture to propose the term "saccharoids" to designate the group of non-fermentable copper-reducing materials present in blood. We believe that most workers who have to deal with this fraction in blood will agree with us that the use of some single term to designate the fraction which we propose to include under the name saccharoids, is desirable. There are several arguments in favor of adoption of a single term to include this group of substances. The chief of these are: (1) the use of a single term eliminates the very awkward and long phrases commonly employed, which are quite undesirable both in writing and in speaking of the group of compounds; and (2) unless the group is more definitely characterized than has hitherto been done it appears likely that various workers will soon mean quite different fractions, though still using the same original and awkward phrases. Thus we note that Herbert and Bourne (2) have sought to make the fraction of non-fermentable reducing materials as large as possible, through adopting the use of a ferricyanide method in studying this group. Ferricyanide in alkaline solution is strongly reduced by many substances which do not appreciably affect carbonate-containing copper reagents. We may mention here uric acid, protein, and simple phenols. It is obviously not desirable to include such compounds, as they have no significance whatever in connection with the problem being investigated. The use of a definite term, and which is as definitely characterized as may be, should serve to make comparable the results reported

by various investigators. The use of the term selected to characterize the non-fermentable copper-reducing fraction will probably be short-lived, as it will naturally be abandoned as soon as the compounds covered are definitely known. The term saccharoids seems to us to be the most satisfactory of the terms which we have considered.¹ The word saccharoid (sugar-like) has long been in use as an adjective, and use of the word as a noun does not seem objectionable. It is easily pronounced, and indicates as well as might readily be done, the fact that these compounds in blood behave in one way like sugars. Later in this paper we shall indicate more closely what fraction we feel should be included under the term saccharoids.

New Copper Reagent for Determination of Sugar in Blood

In previous articles (1, 3) we have called attention to the advance in sugar analysis which is made possible through addition of sodium sulfite to certain copper reagents. Addition of sulfite results in a marked increase in the amount of copper reduced by a given amount of glucose, together with a marked increase in the specificity of the reagent for glucose in blood and urine. In using sulfite heretofore in connection with sugar determination we have started upon the assumption that a reagent would be best which was so made as to yield an essentially perfect blank. This work resulted in the reagent described in 1928. Subsequent studies have shown that it was a serious mistake to assume that a reagent would be best which showed absolutely no blank. Indeed we have found that it is only through the use of a solution showing slight autoreduction that the truly remarkable effects of sulfite in connection with sugar analysis may be realized.

The new reagent has the following simple composition.

Sodium carbonate (anhydrous)	15 gm.
Alanine	3 "
Rochelle salt	2 "
Copper sulfate (crystallized)	3 "
Distilled water to make 500 cc.	

The alanine, Rochelle salt, and copper sulfate should be weighed accurately. The sodium carbonate may be weighed more roughly.

¹ Professor L. B. Mendel suggested the use of the term saccharoids in this connection.

Dissolve the carbonate, alanine, and Rochelle salt in 300 to 400 cc. of distilled water. Dissolve the copper sulfate separately in 50 to 75 cc. of water and add this to the other solution with constant stirring. Dilute the deep blue solution to 500 cc. and mix. This reagent may be kept for some weeks without appreciable deterioration.² When used without addition of sulfite the new reagent gives about as much color from an equal weight of glucose as does the Folin-Wu reagent, and yields figures for whole blood filtrates quite comparable with those obtained through use of the old Folin-Wu method (Table II). When used with a small amount of sulfite ($\frac{1}{4}$ as much as we employed with the 1928 reagent) the character of the reagent is changed to a remarkable degree. The total color yielded with glucose is very greatly increased and reduction due to the saccharoids in blood falls to a value which is essentially negligible. The following results may be cited to illustrate the effect of the bisulfite upon the new reagent. When the reagent is employed without bisulfite, and with a glucose standard corresponding to 20 mg. per cent, set at 15 mm., the color due to reduction by 2 cc. of fermented filtrate reads 16.0 mm., thus giving a value of 18.6 mg. per cent for the saccharoid content of this sample of blood. When the determination was repeated with bisulfite in the reagent the filtrate read 23 mm. against a 10 mg. per cent standard, indicating a saccharoid content for the blood of 6.5 mg. per cent. This value is as low as that obtained with the Folin-Wu reagent upon the zinc (Somogyi) filtrate from this sample of blood. Later in this paper we shall describe the interesting behavior of the new reagent with glutathione.

Regular (Macro) Method for the Determination of Blood Sugar

Solutions Required—(1) A standard solution containing 0.1 mg. of glucose per cc., (2) the new copper reagent, (3) a 1 per cent solution of sodium bisulfite,³ (4) color reagent.⁴

² If kept cool the mixed solution will keep without appreciable deterioration for at least 6 to 8 weeks. During warm weather it deteriorates much more rapidly, as indicated by a marked increase in the blank. We are at present studying the questions involved in keeping the solution without deterioration.

³ The 1 per cent bisulfite solution is conveniently kept in a 100 cc. dropping bottle. The solution should be prepared fresh about once in 3 weeks.

⁴ The color reagent is the one previously described ((1) p. 462). It is

A volume of the sugar reagent which will be used up within 1 or 2 days is measured into a glass cylinder and 1 drop of the 1 per cent solution of sodium bisulfite is added for each cc. of the reagent, or 1 cc. of bisulfite solution for each 20 cc. of reagent.⁵ The solution is then mixed.

2 cc. each of the standard glucose solution and of the 1:10 blood filtrate are measured into Folin-Wu sugar tubes, and 2 cc. of the copper reagent (containing bisulfite) are added to each. The contents of the tubes are mixed by lateral shaking and the tubes placed in vigorously boiling water for $5\frac{1}{2}$ to 6 minutes. The tubes are then placed in cold water for 1 to 2 minutes, and to each tube are then added 2 cc. of the color reagent. The contents are mixed by vigorous lateral shaking, and after about 1 minute water is added to each tube to the 25 cc. mark. The contents of each tube are thoroughly mixed by repeated inversion and shaking, and the colored solutions read in the colorimeter, preferably within 10 minutes after dilution. The calculation of results is the same as in the older (Folin-Wu, etc.) methods.

In Table I are presented figures obtained through use of the new method with twenty different samples of human blood. Comparative analyses are also given upon the Somogyi zinc filtrates and upon fermented samples of blood filtrate with and without addition of glucose. The blood samples were from hospital and ambulatory clinic cases. In very few instances do the sample numbers represent a specimen from one individual. So many determinations were made that it was nearly always necessary to mix two or more samples to obtain enough blood for the complete analyses.

prepared as follows: Place 150 gm. of pure molybdic acid (which must be essentially free from ammonia) in a large Erlenmeyer flask and add 75 gm. of pure anhydrous sodium carbonate. Add water in small portions, with shaking, until about 500 cc. have been added. Shake thoroughly and heat the mixture to boiling or until nearly all of the molybdic acid has dissolved. An appreciable amount of insoluble material remains, which is filtered off. The residue on the filter is washed with hot water until the total volume of filtrate and washings is about 600 cc. Add 300 cc. of 85 per cent phosphoric acid to the total filtrate, cool, and dilute to 1 liter.

⁵ The copper reagent to which bisulfite has been added may be used the 2nd day after the bisulfite has been added, but should not be used later than this.

The figures presented in Table I show that the new method, when applied directly to "complete" (tungstomolybdic acid) filtrates from whole blood, yields results which are correct within an average of about 3 to 5 mg. per cent. The results are nearly the same whether the analyses are made upon a filtrate (tungstomolybdic acid) containing all the saccharoids originally present in blood, or whether zinc filtrates (Somogyi (4)) are used in which the saccharoids (as measured by other sugar reagents) are present in only about one-third to one-fifth the concentration found in the tungstomolybdic acid filtrates. The seventh column of Table I, where figures are reported for fermented filtrates plus added glucose, illustrates this point. The fifth column of Table I also shows that figures for original blood sugar differ by only about 2 mg. per cent whether tungstomolybdic acid or zinc filtrates are employed. When the work was nearly completed we found that the sugar values with the new reagent are frequently slightly depressed by the zinc contained in the Somogyi filtrates. When this zinc is removed by the addition of a trace of solid sodium carbonate, with shaking and centrifuging prior to analysis, results on the two filtrates are often very nearly identical. In this connection it should be pointed out that the Folin-Wu reagent is definitely affected by the zinc contained in the Somogyi filtrates, and the zinc must be removed prior to analysis with the Folin-Wu reagents. Somogyi, in his discussion of the zinc filtrates, cautioned against the use of "unbuffered" sugar reagents, thus implying that acidity of the zinc filtrates is responsible for the inaccurate figures. The zinc filtrates are, however, quite as nearly neutral as are tungstomolybdic or tungstic acid filtrates. The zinc salt itself undoubtedly exhibits an inhibiting effect upon many of the copper reagents. The Somogyi copper reagent is practically unaffected.

The results reported in Table I show that we have not been able to substantiate fully the findings reported by Somogyi in relation to the zinc precipitation. Somogyi's work has conveyed the impression that the zinc filtrates are in nearly all instances entirely free from non-fermentable reducing materials (saccharoids). Employing the Folin-Wu method we have always found saccharoids present in zinc filtrates, the figures ranging from 5 to 9 mg. per cent. With the writer's new reagent saccharoids may also be detected in the zinc filtrates.

TABLE I

Figures for the Sugar Content of Blood as Determined upon Tungstomolybdic Acid and Zinc Filtrates from Whole Human Blood by the New and the Folin-Wu Reagents

Results are expressed as mg. of glucose per 100 cc. of blood.

Sample No.	Method of precipitation	Original filtrate		Fermented filtrate			
		New method	Folin-Wu method	New method	Folin-Wu method	Plus 100 mg. per cent glucose	
						New method	Folin-Wu method
1	Tungstomolybdic acid	94	113	7	20	105	120
	Zinc	94	95	4	7	103	106
2	Tungstomolybdic acid	75	91	6	16	103	118
	Zinc	72	74	4	5	101	105
3	Tungstomolybdic acid	105	123	6	25	103	120
	Zinc	104	107	4	6	104	106
4	Tungstomolybdic acid	107	117	7	24	104	117
	Zinc						
5	Tungstomolybdic acid	142	168	7	22	106	121
	Zinc	139	144	4	6	105	109
6	Tungstomolybdic acid	150	176	8	28	106	128
	Zinc	147	153	6	8	104	108
7	Tungstomolybdic acid	92	108	8	19	101	116
	Zinc	90	95	7	9	102	104
8	Tungstomolybdic acid	125	145	8	23	105	124
	Zinc	123	128	5	7	104	107
9	Tungstomolybdic acid	180	214	7	31	102	136
	Zinc	181	183	6	8	101	105
10	Tungstomolybdic acid	75	85	5	9	101	108
	Zinc						
11	Tungstomolybdic acid	187	209	7	19	105	123
	Zinc						
12	Tungstomolybdic acid	156	180	6	28	100	131
	Zinc	153	153	4	6	103	106
13	Tungstomolybdic acid	63	77	7	11	103	116
	Zinc	61	65	5	7	103	104
14	Tungstomolybdic acid	135	154	6	22	98	131
	Zinc	133	138	4	7	102	106
15	Tungstomolybdic acid	93	115	7	24	104	129
	Zinc	91	94	6	7	102	108
16	Tungstomolybdic acid	115	132				
	Zinc	113	120				
17	Tungstomolybdic acid	128	156				
	Zinc	125	130				

TABLE I—*Concluded*

Sample No.	Method of precipitation	Original filtrate		Fermented filtrate			
		New method	Folin-Wu method	New method	Folin-Wu method	Plus 100 mg. per cent glucose	
						New method	Folin-Wu method
18	Tungstomolybdic acid	150	176	5	29	102	121
	Zinc	145	151	5	7	101	105
19	Tungstomolybdic acid	105	123	7	25	105	120
	Zinc						
20	Tungstomolybdic acid	82	114	6	32	104	133
	Zinc						

In connection with the problem of determining minimal non-sugar reducing values in blood filtrates, there are some points which merit discussion. Determination of saccharoid values of 12 mg. per cent or over in fermented filtrates may readily be made with most of the copper reagents with a fair degree of accuracy. When, however, the values fall appreciably below 10 mg. per cent, special precautions must be taken. The blanks which are yielded by the Somogyi and to a somewhat lesser extent by the Folin-Wu reagent introduce an intolerable source of error where fermented filtrates are used without preliminary concentration. Thus, using the Somogyi reagent (5) and employing the best tartrate we have been able to find (Kahlbaum's), one obtains a blank equal to more than the color given by 10 mg. per cent of glucose. The proportionality of the solution at such low concentrations of glucose is also quite poor. A standard corresponding to 20 mg. per cent in blood, read against a 10 mg. standard, will indicate only about 13 to 14 mg. per cent for the former solution. Where such conditions obtain it is obvious that special precautions must be observed. We believe that the safest procedure where reagents are used which show a high blank, and determinations are to be made involving very low reducing values, is to add glucose to the filtrate.⁶ This is very simply done as follows: To 9 cc. of the fermented filtrate, using an Ostwald pipette, add 1 cc. of 0.1

⁶ An alternative procedure which we have frequently employed is concentration of the fermented filtrate through boiling. No reducing material is destroyed through this process.

per cent glucose solution. Employ for a standard a portion of a solution made by measuring 9 cc. of distilled water with the same pipette used for measuring the filtrate, and 1 cc. of the 0.1 per cent glucose solution. To obtain the saccharoid content, add 10 per cent of the value obtained above unity. Obviously from the practical standpoint of being able to correct so that one obtains the true sugar value for the blood, this procedure is theoretically better than separate determination of the saccharoid value, since the saccharoid value may not be strictly additive, and proportionality at the low sugar levels is not very close. Providing the zinc has been removed from the filtrates, and using the Folin-Wu reagent, the above procedure will, according to our experience, readily demonstrate saccharoids in every sample of zinc filtrate from blood.

In the writer's new method, and with bisulfite in the quantity indicated, the color obtained in a blank is equal to slightly less than the color given by a 5 mg. per cent standard. By using a standard reading *very close to the unknown*, one can make quite satisfactory determinations of reduction values down to about 5 mg. per cent. In determining the saccharoid values reported in Table I the unknowns were always read against standards from which they varied by not more than 1 to 2 mm.

It will be noted that figures for sugar by the new technique average about 2 mg. per cent higher for the complete tungstomolybdic acid filtrates than for the zinc filtrates. Much the same relationship holds for the fermented filtrates to which 100 mg. per cent of glucose was added. From the figures obtained for glucose added to fermented filtrates it is necessary to subtract a correction of about 2 mg. per cent (1 mg. for the blank with the yeast and about 1 mg. for reducing material from the filter paper employed). Thus it will be seen that the figures yielded by the new method directly upon tungstomolybdic acid filtrates are accurate to within 2 to 4 mg. per cent. This is quite close to the limits of accuracy of the colorimetric method. We are doubtful whether essentially more accurate figures for blood sugar can be obtained by any other procedure, however elaborate. Redetermination of the sugar in a fermented filtrate to which glucose has been added might theoretically yield more accurate figures. Yet even here the gain would be slight since the blank for the

yeast and filter paper employed is somewhat variable. The new method may be applied to tungstomolybdic or tungstic acid filtrates or to any filtrate which does not contain substances which adversely influence the reduction by sugar.

Distribution, Nature, and Determination of Saccharoids (Non-Fermentable Copper-Reducing Fraction) in Blood

Van Slyke and Hiller (6) first adequately studied the non-fermentable reducing fraction in blood by comparative studies of the reducing power of blood filtrates by various sugar methods before and after brief but complete fermentation with yeast. The accuracy of results obtained through such studies has been increased through the use of washed yeast for the fermentation (Somogyi, Benedict).

Folin and Svedberg (7) studied the occurrence and distribution of saccharoids in the blood and reported that the non-sugar reducing value of plasma is as great as that of whole blood. Somogyi (8) recently reported an average figure for the saccharoid content of plasma of 5 mg. per cent.

Herbert and Groen (9) reported a study of blood sugar values by various precipitation and reduction methods. Their figures show that while the greater part of the saccharoids is to be found in the corpuscles, a very considerable portion of this non-sugar reduction is also to be found in the plasma, since the reduction values for plasma are higher by the copper methods upon tungstic acid filtrates than upon zinc filtrates.

Though both Herbert and collaborators and Folin and collaborators had clearly shown that a considerable proportion of the saccharoids in blood exists in the plasma, both of these investigators, when they later favored the use of unclaked blood for analysis, reported that the non-sugar reducing materials are essentially absent from the unclaked blood filtrates. Since the same precipitating agent (tungstic acid) is employed with unclaked blood, it is evident that if, as these investigators had shown, saccharoids are present in plasma, they are also present in the filtrates from unclaked blood. We may perhaps explain the low values obtained by Herbert and by Folin for saccharoids in unclaked blood by assuming that the non-fermentable reducing material present in plasma is different from that in the corpuscles and does not diffuse

into or out from the corpuscles. If this is the case it is obvious that the saccharoid values for unlaked blood would be very much lower than for plasma, and might, in many instances, be so low as to escape detection unless especially careful work was done upon this point. When one measures 100 cc. of unlaked blood for analysis one is really measuring 100 cc. of a solution of the freely diffusible substances present, but only (on an average) 58 cc. of a solution of substances which are present in plasma but not in corpuscles, 0 volume of a solution of substances confined to the corpuscles, and different volumes of solutions of the compounds which are unequally distributed between corpuscles and plasma. It is this rather anomalous situation which has probably been responsible for the very low saccharoid values reported for the filtrates from unlaked blood. If, as seems probable from the figures so far reported, the plasma saccharoid content is not diffusible and is considerably lower than that of whole blood, it is obvious that when this figure is reduced to an average of 58 per cent of the plasma content, which occurs when unlaked blood is analyzed, the figures may become so low as to escape notice. It is only on the assumption that the plasma saccharoids are non-diffusible that any explanation seems possible of the wholly contradictory reports by Herbert and by Folin as to the saccharoid content of plasma and of unlaked blood.

In the present work we have made a study of the saccharoid content of only five samples of plasma. The figures obtained have shown such wide variation that they warrant no conclusion. In two of the samples analyzed, the figures for saccharoids were almost as high for plasma as for whole blood, 17 against 18 and 23 against 26 mg. per cent in plasma and whole blood respectively. In the other three samples analyzed the plasma showed a saccharoid content of only 7 to 8 mg. per cent. In view of these widely differing values and in view of the discrepancies in the figures available in the literature, it seems that the question is in need of further very careful study. For the present the only conclusion warranted seems to be that saccharoids are to be found in both plasma and corpuscles in readily detectable quantity, but that in many samples of blood the analysis of unlaked blood by the usual methods may fail to reveal their presence.

Herbert and Bourne (2) have made some surprising interpretations of the very interesting studies which Herbert and collaborators have recently reported. Herbert and Bourne have concluded that the different results for sugar values by different methods "may be explained by the single postulate that the blood analyzed contained a certain definite quantity of reduced glutathione (44-60 mg. per 100 cc. whole blood) present in corpuscles in non-diffusible form." This conclusion is reached in spite of the fact that Herbert and collaborators (9, 10) clearly presented facts which are not compatible with the view that glutathione is the only important constituent of blood responsible for the non-sugar reduction given by blood filtrates. Thus Herbert and Groen had shown marked discrepancies in plasma sugar values by different methods. These same investigators had shown that the Shaffer-Hartmann (Somogyi) copper and iodine method gave very nearly twice as great a reduction value for glutathione (39) as did the Folin-Wu method (20). Herbert and Groen (9, 10) reported for blood filtrates no such order of difference by these two methods. Using the nine blood samples analyzed by Herbert and Bourne by both the Shaffer-Hartmann and the Folin-Wu methods upon tungstic acid (glutathione-containing) and Somogyi zinc (glutathione-free) filtrates as a basis of calculation, we find the following differences by the two methods. Results by the Shaffer-Hartmann method averaged 20.0 mg. per cent higher for the tungstic acid than for the zinc filtrates, while results by the Folin-Wu method averaged 16.4 mg. higher for the tungstic acid than for the zinc filtrates. Had the difference been due solely to glutathione the Folin-Wu method would have shown an average difference of 10.2 mg. per cent, against 20 mg. by the Shaffer-Hartmann method. Thus had the difference by the two methods been due to glutathione alone it should have been 160 per cent of that actually found. This fact, and the differences found by different methods for plasma sugar values, show that glutathione cannot account for even a very large proportion of the saccharoid content of blood.

Everett (11) called attention to the fact that since the Benedict (1928) reagent is not appreciably reduced by glutathione, but is reduced by fermented blood filtrates, these latter solutions contain appreciable quantities of reducing non-sugar substances other

than glutathione.⁷ Like Everett, Herbert and Groen found that the Benedict (1928) reagent is not appreciably reduced by glutathione, but they neglected to take into account the fact, clearly brought out by Benedict when the 1928 reagent was described, that fermented blood filtrates do markedly reduce this reagent, though it is reduced only about one-half as much as is the Folin-Wu reagent.

From the foregoing facts it is evident that a considerable portion of the saccharoid fraction of blood is not represented by glutathione. Until extensive and accurate figures are available for the glutathione content of blood it is not possible to estimate at all closely what proportion of this fraction glutathione represents. We are of the opinion that on the average glutathione accounts for little of the saccharoid content of blood. Our reasons for this view are as follows: The relatively high reduction value given by fermented filtrates with the Benedict (1928) reagent speaks against the assumption that glutathione contributes very largely to the saccharoid fraction. The average figure of 19.9 mg. for the saccharoid content of the twenty samples reported in Table I would require about 100 mg. of glutathione per 100 cc. of blood. There is not satisfactory evidence that blood contains upon an average one-half as much glutathione as this.⁸

⁷ In bringing out this point Everett stated that when Benedict and Newton found that glutathione in the presence of glucose gave no higher reduction figures than glucose alone, they "interpreted their results as the disappearance of the reducing action of a non-sugar substance in the presence of glucose." What Benedict and Newton (12) actually said in this connection is, "This is in accord with the findings reported by one of us that in the presence of much glucose the non-sugar reducing substances of blood do not appreciably affect the Benedict copper solution." Thus it is evident that Benedict and Newton said nothing about the "disappearance" of any reducing action. No statement was made that glutathione alone reduced the copper solution. Tests were being made primarily with the Folin-Wu reagent, and an experiment was tried in which recovery of glutathione added to sheep blood was tried with both the Folin-Wu and the Benedict (1928) reagents. The results obtained with both reagents were stated in the paper, but the interpretation to which Everett objected was his own interpretation rather than that of Benedict and Newton.

⁸ Dr. Helen R. Downes of the Department of Chemistry, Memorial Hospital, New York City, informs me that six human bloods analyzed by the Mason (13) method gave an average glutathione content of 23.1 mg. per

The behavior of the new sugar reagent described in the present paper toward glutathione is interesting and serves to illustrate strikingly the specificity for glucose which may be gained by use of the new technique. When used without bisulfite the new reagent is more strongly reduced by glutathione than is the Folin-Wu reagent. Without bisulfite and with 0.5 mg. of glutathione for the determination,⁹ the new reagent gives a reduction value of 1 mg. of glucose equal to 3.7 mg. of glutathione, against a value of 1 mg. of glucose equal to 5 mg. of glutathione by the Folin-Wu reagent. In the presence of sulfite the reducing power of glucose is greatly increased, *while the actual reduction by glutathione in the presence of sulfite is only about one-half as great as in the absence of sulfite.* Due to the change in opposite direction of the reducing value of the two compounds in the presence of sulfite, the reduction given by glucose in the presence of sulfite is 16.6 times as great as that of an equal weight of glutathione. Thus in the absence of sulfite 100 mg. of glutathione will give a reduction value equal to 27 mg. of glucose, while in the presence of sulfite, 100 mg. of glutathione will give a reduction value equal to 6 mg. of glucose. Such results indicate, as do the results reported in Tables I and II, that the new reagent should be of definite value in biological sugar analysis.

We may add that the decrease in reduction by glutathione in the presence of sulfite is not due to fading of the final color during the determination. Using 0.5 mg. of glutathione, one can see a relatively abundant greenish precipitate of reduced copper in the tube without the bisulfite, and no visible reduction in the tube with the bisulfite. Furthermore, in the new technique the fading of the final color is negligible even with pure solutions of glutathione in the presence or absence of sulfite.

The marked effect of sulfite in altering the reducing power, both

100 cc., maximum 30.8 mg., minimum 18.5 mg. per cent. Such a content of glutathione in blood is not in agreement with the figures reported by Herbert and Bourne (2) for comparative determinations by the Shaffer-Hartmann and the Folin-Wu methods. Until more extensive figures are available for human blood by the Mason method, and until the accuracy of the method has been further tested, we cannot draw definite conclusions concerning the glutathione content of blood.

⁹ The glutathione used was obtained from the Eastman Kodak Company. A Kjeldahl determination yielded a practically theoretical nitrogen content for the sample.

relative and absolute, of glutathione, should afford one of the most specific reactions now available for that compound, and we are utilizing the reaction to study the presence or absence of glutathione in fermented blood filtrates. In accord with the conclusion reached above, preliminary findings indicate that there is not much glutathione in these filtrates, and we feel sure that only a relatively small proportion of the saccharoid content of blood is represented by a compound having the properties of glutathione which has been isolated from yeast.

The nitroprusside reaction and iodine and ferricyanide oxidations cannot, because of their lack of specificity, counterbalance definite findings for blood filtrates which do not accord with the properties of glutathione. It may well be that a cystine complex similar to glutathione exists in blood in appreciable amounts, but if so, its properties may be, and apparently are, quite different from the properties of glutathione.

Because we feel that the saccharoid content of blood deserves investigation we are proposing a simple method by which this fraction may readily be followed in whole blood or in plasma. The procedure consists simply in making determinations upon the same tungstomolybdic acid filtrate by the new technique, with and without addition of bisulfite to the copper reagent. The difference between the two figures obtained will represent the saccharoids in terms of glucose. (The same copper reagent, *i.e.* with and without bisulfite, must of course be employed for both standard and unknown.) Typical results for such determinations upon filtrates from whole blood (human) are presented in Table II. Saccharoid values obtained in this way are very close to those obtained through use of the Folin-Wu reagent and subtraction of the reduction figures obtained after fermentation.

Since saccharoid values are subject to wide variations where different bloods are compared, it would seem well worth while to attempt to correlate these findings with some definite condition of the individual studied. The present method makes it very easy to make such studies. For the sake of securing uniformity in results we suggest that the term saccharoids be essentially limited to results obtained by the new method for their determination. We do not at all suggest that the proposed procedure be the only one applied to study rest reduction in the blood, but

we feel that if results are to be comparable a standard method of procedure should be adopted. The copper reduction methods which involve iodine titration may add values due to oxidation and substitution by iodine, which may contribute to the final result in complex fluids. The iodine and ferricyanide methods are very valuable procedures, but those employing them should clearly recognize that they are definitely less specific for reducing sugar than are the copper colorimetric methods. The relatively high

TABLE II

Showing the Saccharoid Content of Samples of Human Blood as Determined by Difference between Results by the New Technique with and without Addition of Sulfite

Determinations by the Folin-Wu method are included to show that the figures thus obtained closely approximate those by the new technique without sulfite.

Results are expressed as mg. of glucose per 100 cc. of blood.

Sample No.	Sugar		Saccharoids	Sugar by Folin-Wu method
	New method with sulfite	New method without sulfite		
1	187	210	23	209
2	141	157	16	156
3	156	187	31	180
4	84	98	14	100
5	90	107	17	105
6	63	75	12	77
7	68	81	13	82
8	100	125	25	123
9	135	157	22	154
10	120	138	18	146

“sugar” values of glutathione or of phenols by such methods clearly indicate this fact.

New Micro Method for Determination of Blood Sugar Directly on Filtrates from Whole Blood

Slight changes in technique serve to adapt the new sugar reagent to the determination of sugar in small quantities of blood. (0.1 cc. or less). This procedure offers the first highly specific micro method for blood sugar determination. The intensity of color finally read is about as great as in the older macro methods for

blood sugar determinations. The volume of solution (8 cc.) available for the colorimeter reading is ample for satisfactory readings up to a depth of 35 mm. or more, even where the cups are rinsed with the solution, providing a colorimeter is used which employs small (2.5 to 3 cc.) cups. Where large cups only are available, the cups should be rinsed with water and wiped dry before introducing the colored solutions for reading.

Solution for Precipitation of Blood—A dilute solution of acidified tungstomolybdic acid is prepared as follows: Measure 5 cc. of the molybdotungstate solution (14) into a 250 cc. graduated flask. Add about 150 cc. of distilled water, followed by 5 cc. of the 0.62 N sulfuric acid. Dilute to 250 cc. and mix. This precipitating solution should be prepared fresh within 3 to 5 days of its use.

For precipitation of the blood, measure 5 cc. of the precipitating solution into a 15 cc. centrifuge tube. In a pipette calibrated to contain 0.1 cc. measure 0.1 cc. of blood and discharge this vigorously into the solution in the centrifuge tube, and rinse the pipette two or three times with portions of the mixture. Stopper the centrifuge tube, shake vigorously, and centrifuge for 3 minutes at the end of about 1 minute or longer. The same copper reagent is used as in the macro method, but instead of 1 drop of the 1 per cent bisulfite for each cc. of solution, 2 drops should be added. Measure 2 cc. of the clear supernatant blood extract into a Folin-Wu sugar tube, and add 1 cc. of the copper reagent containing the bisulfite, and mix. Add enough benzene to fill the constricted portion of the Folin-Wu tube. The standard solution is prepared by diluting the 0.01 per cent glucose solution 1:5, and is treated just as the unknown. The tubes are heated for exactly 5 minutes in a vigorously boiling water bath, and are cooled for 1 minute (avoid shaking) in a large volume of cold water. 2 cc. of the color reagent are then added, the solutions mixed by lateral shaking, and 3 cc. of water added to each tube. Mix at once by inversion and shaking and read in the colorimeter within 10 minutes.¹⁰

The calculation is the same as in the macro method, except that 2 per cent of the value obtained should be added to correct

¹⁰ Prior to pouring the colored solution into the colorimeter cups it is well to eliminate excess of CO₂ by shaking each tube and connecting it with a suction pump for a few seconds.

for the dilution of the 0.1 cc. of blood to 5.1 cc. instead of to 5.0 cc. The volume of 5 cc. of precipitating solution is used instead of 4.9 cc. because the 5 cc. volume is more readily measured.

It may be noted that in using the micro method 0.05 cc. of blood may be used in place of 0.1 cc. if desired, with 2.5 cc. of the precipitating solution in place of 5 cc. Duplicate determinations cannot then be made, but if care is exercised in the determination, duplicates may be dispensed with. It should be borne in mind, that measurement of 0.05 cc. of blood introduces the probability of considerable error unless the greatest possible care is exercised. A 10 to 20 per cent error in such measurements is not uncommon.

TABLE III

Showing That Results for Blood Sugar by the New Macro and Micro Methods Are Very Similar

Samples of human blood were used.

Results are expressed as mg. of glucose per 100 cc. of blood.

Sample No.	Macro method	Micro method
1	187	182
2	141	144
3	156	160
4	84	84
5	90	88
6	63	57
7	68	66
8	100	98
9	135	131

In bloods very low in sugar content (such as those often obtained after excessive doses of insulin) the initial dilution of the blood should be only half as great (1:25) as with normal or hyperglycemic bloods. In such cases the precipitating solution should contain twice as much of the molybdotungstate solution and 0.62 N sulfuric acid, and 0.1 cc. of blood should be added to 2.5 cc. of this solution. The proportionality of the micro method is practically just as good as that of the macro method. In either case there may be up to a 7 to 8 per cent error where the unknown reads double or one-half of the standard. Table III shows that results by the macro method closely duplicate those by the micro method.

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THE ANALYSIS OF WHOLE BLOOD

III. DETERMINATION AND DISTRIBUTION OF URIC ACID

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Since one of us (1) first described a direct method for the determination of uric acid in filtrates from human blood, it has been clearly recognized that the direct methods yield results which are too high. The error involved is usually from 0.5 to 1 mg. per cent in tungstic acid filtrates from whole human blood. In the case of tungstomolybdic acid filtrates the error is somewhat greater when the direct method is used because, unlike tungstic acid, tungstomolybdic acid permits all of the thioneine present in blood to pass into the filtrate (Benedict and Newton (2)). Though this error is small in absolute amount, the percentage error involved is very considerable.

With the isolation of thioneine, its identification as the substance chiefly or wholly responsible for the high uric acid values in the direct method, and the finding that occasionally excessive quantities of this substance may occur in blood (up to 25 mg. per cent or more), we have felt that from a practical as well as from a theoretical standpoint it was desirable that a simple method for uric acid determination should be developed which would eliminate the errors due to thioneine. We first endeavored to solve the problem through making the color reaction more specific for uric acid. With this end in view Miss Gertrude Gottschall developed in this laboratory a technique which cut down interference due to thioneine to about one-half of what it is when the older technique is employed. The technique involved essentially the use of a dilute phosphotungstic acid reagent as the color reagent, the reaction being carried out in the presence of a considerable excess of acid. The method thus developed fell just short of what we felt would

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be a thoroughly dependable technique, certain to yield essentially correct uric acid values under even the most adverse conditions.

The procedure for uric acid determination now proposed is based upon the observation which we previously reported (3) that when silver chloride is precipitated in the presence of a strong acid in a solution containing thioneine, the thioneine is carried down in the precipitate. Since Folin and Wu showed that uric acid is liberated from its silver combination by treatment with a strongly acid solution of a chloride, we were led to expect that a complete separation between thioneine and uric acid might be effected by a single silver precipitation in a strongly acid medium. The results here have been all that we hoped for. The technique described below will, by means of a single instantaneous precipitation and about $\frac{1}{2}$ minute of centrifugation, effect a quantitative separation between mixtures of thioneine and uric acid containing 0.25 mg. per cent of uric acid and 2.0 mg. per cent of thioneine or 2.0 mg. per cent of each of these compounds. We have not tried other quantities of the compounds, because there seemed to be no object in carrying the observations any further.

As the figures in Table I show, from the standpoint of specificity, the new technique has essentially the advantages of the old Folin-Wu precipitation and decomposition, yet the new procedure is almost as simple as the direct method. Acid chloride and silver solutions are added to the filtrate in place of the diluting water, and after a few seconds centrifugation the solution is ready for analysis. The details for the macro modification of the new technique follow.

New Macro Method for the Determination of Uric Acid in Blood

Solutions Required

1. Solutions for precipitating the blood proteins are prepared as described in the first paper of this series (4).

2. The arsenotungstic acid color reagent previously described (1). This is prepared as follows: 100 gm. of pure sodium tungstate are placed in a liter flask and dissolved in about 600 cc. of water. 50 gm. of pure arsenic pentoxide are now added, followed by 25 cc. of 85 per cent phosphoric acid, and 20 cc. of concentrated hydrochloric acid. The mixture is boiled for 20 minutes, cooled, and diluted to 1 liter. The reagent appears to keep indefinitely.

3. A 5 per cent solution of sodium cyanide, containing 2 cc. of concentrated ammonia per liter. This solution improves during the first 2 to 3 weeks after its preparation, but should not be used after 6 to 7 weeks.

4. A standard uric acid solution containing 0.02 mg. of uric acid in 5 cc., corresponding to 4 mg. of uric acid per 100 cc. of blood, and containing hydrochloric acid. This standard solution is prepared from the stock phosphate standard solution (5) as follows: 10 cc. of the phosphate standard solution (containing 2 mg. of uric acid) are measured into a 500 cc. flask and diluted to about 400 cc. with water. 5 cc. of concentrated hydrochloric acid are then added and the solution is diluted to 500 cc. and mixed. This solution should be prepared fresh about once a week.

5. A solution containing 3 gm. of lithium chloride¹ and 20 cc. of concentrated hydrochloric acid per liter.

6. A solution containing 11.6 gm. of silver nitrate per liter.

Procedure—Transfer 5 cc. of the 1:10 blood filtrate to a 15 cc. centrifuge tube. Add 2.5 cc. of the acid lithium chloride solution and mix by inversion of the tube. Add 2.5 cc. of the silver nitrate solution, and shake the contents of the tube thoroughly (using a tight rubber stopper). Centrifuge for about $\frac{1}{2}$ minute or longer and pour off all the clear supernatant liquid² into a test-tube.

¹ 4 gm. of sodium chloride may be used in place of the 3 gm. of lithium chloride, but turbidity is more apt to develop in the presence of the former salt. We strongly advise that sodium oxalate or lithium oxalate (as suggested by Folin) be used as an anticoagulant in all blood samples. The use of either of these salts in place of potassium oxalate minimizes the danger of turbidity in the uric acid and the creatinine determinations. Potassium oxalate offers no advantages, save that it is less expensive than the lithium salt. Sodium oxalate, though less soluble than the lithium or potassium oxalate, has so high a solubility factor as compared with calcium oxalate that it is entirely satisfactory as an anticoagulant. Sodium oxalate should be used in very finely powdered form, or a solution of the salt may be dried upon the walls of the vessel in which the blood is collected. Under such conditions the sodium salt is highly effective, and its lower solubility tends to prevent dissolving such excessive quantities of an oxalate in the blood as to interfere with the proper precipitation of the proteins.

² The supernatant solution will usually be almost perfectly clear. Even though a distinctly opalescent supernatant fluid is obtained (due to the presence of a trace of colloidal silver chloride) the accuracy of the results is not impaired. Opalescent solutions will become perfectly clear when the cyanide solution is added.

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Transfer 5 cc. of the standard uric acid solution to another tube, and add 5 cc. of water. Add 4 cc. of the sodium cyanide solution to each tube followed by 1 cc. of the color reagent. Each tube is inverted once immediately after addition of the reagent and placed at once in a boiling water bath, where the tubes are left for 3 minutes after immersion of the last tube. The tubes are then removed from the water bath and allowed to stand at room temperature for about 2 minutes, after which they are read in a colorimeter while still warm or even hot. The reading of the standard divided by the reading of the unknown is multiplied by 4 to obtain the mg. of uric acid in 100 cc. of blood.

We believe that discovery of the fact that the final colored solutions may be read while very warm with the same results which are obtained if one cools the solutions to room temperature before reading, represents a distinct advance toward avoiding development of the turbidity which troubles many analysts during uric acid determinations. Even with bloods containing excessive quantities of potassium oxalate turbidity very seldom develops until after the solutions have become cool.

In Table I are recorded analyses of tungstomolybdic acid filtrates from twenty-two samples of human blood. In twelve of the samples separate analyses of plasma were also made. In each case the uric acid was determined by three methods, *viz.* the direct method of Benedict (1), the new procedure, and a method using the Folin-Wu precipitation with acid silver lactate solution, and decomposition of the precipitate with acid sodium chloride solution, prior to developing the color just as in the direct method. It will be noted that for the whole blood results by the old direct method average more than 1 mg. higher than by the new technique. Results by the new technique approach very closely the figures obtained by the Folin-Wu indirect precipitation method. In the plasma, which is practically free from thioneine, it will be noted that results by all three methods agree very closely.

Single analyses of pig and of sheep blood are included in Table I. These serve to illustrate the large differences which may be expected between results by the old direct and the new techniques in samples where the thioneine is high and the uric acid content almost negligible. The uric acid figures recorded for these

TABLE I

Comparative Figures for Uric Acid Content of Human Whole Blood and Plasma by the Direct, the Folin-Wu, and the New Methods

Figures represent mg. of uric acid per 100 cc. of blood.

Sample No.	Whole blood			Plasma			$\frac{\text{Corpuscle uric acid}}{\text{Plasma uric acid}} \times 100$
	Direct	New method	Folin-Wu (indirect)	Direct	New method	Folin-Wu (indirect)	
1	4.2	3.3	3.1	3.8	3.7	3.7	76
2	4.4	3.3	3.1	3.7	3.7	3.6	74
3	4.5	3.5	3.7	4.2	4.2	4.3	59
4	4.6	3.5	3.5	4.1	4.3	4.3	57
5	4.6	3.2	3.0	3.9	3.4	3.6	83
6	4.7	3.7	3.4	4.1	4.0	4.0	81
7	4.8	3.8	3.6	4.0	4.2	4.1	77
8	4.9	4.3	4.1	4.8	4.7	4.6	77
9	4.8	3.8	3.5	3.9	3.9	3.4	91
10	5.0	3.6	3.4	4.5	4.4	4.5	58
11	5.4	4.8	4.7	5.5	5.5	5.6	70
12	5.1	4.2	4.2	4.9	4.8	5.1	68
13	4.0	3.1	2.8				
14	4.2	3.0	3.0				
15	4.5	3.4	3.2				
16	4.6	3.6	3.6				
17	4.8	3.9	3.7				
18	4.8	4.3	4.0				
19	4.9	4.1	3.9				
20	5.9	5.2	5.2				
21	6.0	4.6	4.6				
22	6.5	5.0	5.0				
Average ..	4.9	3.8	3.7	4.2	4.4	4.2	72
23*	2.3	0.8	0.6				
24†	5.9	0.8	0.7				

* Determination made on sheep blood.

† Determination made on pig blood.

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animal bloods by the new method are probably not very accurate, but it is certain that the uric acid content does not exceed the figure given. Because of the color due to the blank, blood containing less than about 1 to 1.5 mg. per cent of uric acid should be diluted 1:5 rather than 1:10, prior to analysis.

In connection with the samples of blood for which analyses are reported both for whole blood and plasma, we have calculated the corpuscle uric acid in terms of percentage of the plasma uric acid. The results show from 57 to 92 per cent as much uric acid in 100 cc. of corpuscles as in 100 cc. of plasma, with an average figure of 72 per cent. Since the corpuscle volume was not determined, but was assumed to be 42 per cent, the distribution figures obtained may well be somewhat inaccurate. Nevertheless these figures agree much more closely with those reported for the distribution of uric acid in blood by Theis and Benedict (6) and Wu (7), than they do with the distribution figure of 22 per cent reported recently by Folin and Svedberg (8). In this connection we must raise the question as to whether the technique employed by Folin and Svedberg will indeed indicate correctly the distribution of blood constituents other than those which are freely diffusible between corpuscles and plasma. Folin and Svedberg obtained figures for the blood constituents for plasma and for unlaked blood, and upon the basis of these figures calculated the distribution between plasma and corpuscles. It appears to us that such a calculation is based on the assumption that although the equilibrium obtaining in whole blood may show a very unequal distribution of certain constituents between plasma and corpuscles, yet when the hypertonic sulfate solution and precipitating reagents are added to the unlaked blood, an absolutely equal distribution of these constituents takes place. Otherwise there is no basis for calculating distribution by comparative figures from plasma and *unlaked* blood. There would appear to be no grounds for assuming that the addition of the diluting and precipitating solutions causes such a change in the permeability of the wall of the corpuscles as to cause complete diffusion of constituents which were previously unequally distributed between the corpuscle and its surrounding medium.

It seems quite possible that Folin and Svedberg meant by distribution, as they employed the term, not the total amount of any constituent in the corpuscles as compared with the plasma, but

what might be termed the diffusible portion of this constituent in the corpuscles. The phraseology of Folin and Svedberg in connection with uric acid distribution would not seem to admit of such an interpretation, but in their discussion of the distribution of amino acids between corpuscles and plasma they appear to mean concentration of a *diffusible* product in the corpuscles rather than of the total amount present. If, however, Folin and Svedberg were attempting to determine not absolute distribution, but distribution of diffusible fractions, it would have been clearer had they so stated in relation to uric acid as well as to amino acids.

If one wished to study distribution on a basis of diffusibility it would seem that dilution of the unlaked blood should be carried out with an isotonic, rather than a hypertonic salt solution. Even then it is very doubtful whether the results obtained could be interpreted as obtaining *in vivo*. Another factor which complicates such studies is the question of the diffusibility of some of the plasma constituents into the corpuscles. In the preceding paper we offered evidence to show that the saccharoids of plasma are probably not freely diffusible into the corpuscles. Some amino acids of the plasma may not diffuse into the corpuscles. Theis and Benedict (6) reported that uric acid added to whole blood failed, in many cases, to penetrate the corpuscles.³ If one is to study the *diffusible* constituents of corpuscles it would seem that the proper basis for such studies would be to dialyze corpuscles against pure isotonic sodium chloride solution, or perhaps better, against plasma which had been dialyzed against isotonic sodium chloride solution. In any event it is obvious that the question of studying distribution by means of a comparison of figures for plasma and unlaked blood is far from a simple straightforward proposition. We feel that a more definite basis for the conclusions of Folin and Svedberg is necessary, and that these investigators have perhaps been too severe in their criticisms of previous work in the field of the distribution of non-protein constituents in the blood.

New Micro Method for the Determination of Uric Acid in Blood

Through slight modifications the new technique readily lends itself to the accurate determination of uric acid in small amounts of blood.

³ This possibility should be taken into account in connection with studies of the recovery of uric acid added to unlaked blood.

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The reagents required and the procedure are as follows:

Solutions Required

1. *Dilute Precipitating Solution*—Transfer 10 cc. of the tungstomolybdic acid solution (4) to a 250 cc. flask. Dilute to about 100 cc. with water. Add 10 cc. of the 0.62 N H_2SO_4 solution, make to volume, and mix. This solution may be used for 3 to 5 days after mixing.

2. *Lithium Chloride Solution*—Dissolve 1.5 gm. of lithium chloride in water, add 10 cc. of concentrated hydrochloric acid, and dilute to 250 cc.

3. *Silver Nitrate Solution*—Dissolve 5.8 gm. of silver nitrate in water and dilute to 250 cc.

4. *Uric Acid Standard*—This consists of 0.8 mg. of uric acid in 500 cc., corresponding to 4 mg. of uric acid per 100 cc. of blood. Measure 4 cc. of stock uric acid phosphate solution and 300 cc. of water into a 500 cc. volumetric flask. Add 5 cc. of concentrated hydrochloric acid and dilute to 500 cc.

5. *Sodium cyanide solution* as employed in the macro method.

6. *Uric acid color reagent* as employed in the macro method.

Procedure—Transfer 5 cc. of the dilute precipitating solution to a 15 cc. centrifuge tube. With a pipette graduated to contain 0.2 cc., measure 0.2 cc. of blood and discharge this vigorously into the precipitating solution in the centrifuge tube. Rinse the pipette twice with the precipitating solution. Care should be taken not to draw the blood or the precipitating solution much above the mark. Stopper the tube and shake it thoroughly. Let stand for about 2 minutes, then centrifuge for 3 minutes or longer, and transfer 4 cc. of the clear supernatant fluid² to another 15 cc. centrifuge tube. Add 1 cc. of the lithium chloride solution, mix, and add 1 cc. of the silver nitrate solution. Mix thoroughly. Centrifuge and transfer the supernatant liquid to a test-tube. Transfer 4 cc. of the uric acid standard to another tube and add 2 cc. of water. Add 2 cc. of the sodium cyanide solution and 0.5 cc. of the color reagent to each tube. Mix and heat the tubes in boiling water for 3 minutes. Then remove the tubes from the boiling water and allow to stand at room temperature for about 2 minutes. Read in a colorimeter while still very warm. The solutions may be read when cold, but turbidity is then much more likely to develop.

We have carried out numerous comparative determinations upon blood samples by the macro and micro modifications of the new technique. It is needless to present a table showing the comparative figures obtained, since the results by the two methods agree quite as closely as duplicate determinations by either method alone upon the same sample of blood. The accuracy of the results by the micro method is essentially determined by the care and exactness with which the small volume of blood is measured.

In making the calculation it may be assumed that the standard employed is equivalent to 4 mg. of uric acid per 100 cc. of blood, when equal volumes of standard and blood filtrate are employed. The calculation is therefore like that in the macro method; *i.e.*, the reading of the standard divided by the reading of the unknown is multiplied by 4 to give the number of mg. of uric acid in 100 cc. of blood. Add a correction of 4 per cent of the amount of uric acid found because of the dilution to 5.2 cc., instead of to 5.0 cc.

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**PROCEEDINGS OF THE AMERICAN SOCIETY OF
BIOLOGICAL CHEMISTS**

TWENTY-FIFTH ANNUAL MEETING

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THE SOURCE OF ENERGY OF NERVE ACTIVITY

By ETHEL RONZONI

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School of Medicine, St. Louis)*

The energy for the irritable and contractible mechanisms of muscle is derived mainly from the breakdown of creatinephosphate which is restored by energy derived both from oxidation and from the formation of lactic acid. The energy of nerve activity is but a small fraction of that of muscle and the 3 to 4 mg. per cent of phosphorus, indistinguishable by its lability from creatinephosphate, would be sufficient to account for its activity. Nerve and muscle are so much alike in their physiological properties that one is inclined to look for the same mechanism in both; that is, a breakdown of creatinephosphate followed by a restoration from energy derived from lactic acid formation anaerobically and oxidation aerobically.

While this oxidation mechanism has been proved for nerve as for muscle, Gerard and Meyerhof failed to demonstrate a lactic acid mechanism in the absence of oxygen, although they find the resting nerve capable of producing lactic acid anaerobically.

In muscle the energy from lactic acid formation can maintain the activity by a restoration of creatinephosphate in the absence of oxygen. On the other hand, oxidation restoration can take place in the absence of lactic acid formation. In the absence of both, the activity stops with the complete breakdown of creatinephosphate.

Our experiments show that nerves treated with monoiodoacetic acid, stimulated twice per second anaerobically, failed to conduct an impulse after a period of 1 hour when the untreated nerve stimulated under the same conditions showed only slight depression. Oxygenation of the treated nerve restored the original amplitude, rate of conduction, and refractory period. This would indicate that in normal nerve, under anaerobic conditions, activity may in part be maintained by a mechanism inhibited by mono-

iodoacetic acid, presumably lactic acid formation. In oxygen iodoacetic acid has no detectable effect over a period of time necessary to completely depress the anaerobic nerve. That is, oxidation is sufficient to carry on the activity of nerve when lactic acid formation is suppressed, whereas in muscle the lactic acid mechanism alone allows the muscle to carry on for a longer period of time than does oxygen alone.

On the basis of these considerations it is possible to argue that nerve and muscle are alike in having as a source of energy for restoration both a lactic acid and oxidation mechanism, the former predominating in muscle, the latter in nerve.

The assumed oxygen reserve of nerve for whose existence there is no conclusive evidence would then become a creatinephosphate reserve whose utilization during lack of oxygen can be only partly compensated by a poorly developed lactic acid mechanism too slight to measure directly.

THE RELATIVE IMPORTANCE OF THE CHEMICAL AND THE REFLEX CONTROL OF RESPIRATION IN THE MECHANISM OF CARDIAC DYSPNEA

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The mechanism of orthopnea and that of dyspnea produced by slight exertion have been studied in a group of patients with various types of cardiac disease. In an orthopneic subject the shift from the recumbent to the sitting posture was not usually accompanied by significant changes in the carbon dioxide content, carbon dioxide tension, pH, or oxygen content of the arterial blood or of the blood from the internal jugular vein. The difference between the oxygen content of blood from these two vessels was the same as that found in normal subjects and was unaltered by change of posture. Therefore, orthopnea is probably not due to decreased cerebral blood flow. Increased vital capacity, decreased ventilation, and decreased respiratory rate were usually observed in the sitting as compared to the recumbent posture.

Observations made on dogs showed that, when the vagus nerves were intact, artificial diminution in vital capacity produced by

pneumothorax, by introducing Ringer's solution into the lungs or by distending the vessels of one of the lungs with blood, caused increased respiratory rate and increased ventilation. No significant changes in the pH or the gases of the arterial blood or of the venous blood from the brain were observed until the decrease in vital capacity was of marked degree, whereas relatively slight decrease in vital capacity was accompanied by increase in respiratory rate and ventilation. After bilateral vagotomy reduction of vital capacity did not change the breathing until decreased oxygen content or increased acidity of the blood occurred.

Dyspnea produced by mild exertion was also found to be unassociated with significant changes in the pH, carbon dioxide content or tension, or oxygen content of the venous blood from the arm, the blood from the internal jugular vein, or the arterial blood. It is evident that this type of dyspnea is also of reflex nature but further studies are necessary in order to determine its nature more precisely.

Similar findings have been made on normal subjects and again, with *mild* exercise, chemical alterations in the blood were absent. It therefore appears probable that orthopnea and cardiac dyspnea at rest (aside from Cheyne-Stokes respiration and cardiac asthma) are due to alterations in the Hering-Brauer vagal reflex from the lungs. It seems probable that the nervous regulatory mechanism is more delicate than the chemical, and that the former is largely responsible for the changes in ventilation which occur with the usual activities of life, aside from severe exertion.

IS UNIRRADIATED ERGOSTEROL ABSORBABLE?

BY RUDOLF SCHÖNHEIMER

(*From the University of Freiburg, Freiburg, Germany*)

Previous experiments showed that all kinds of plant sterols are not absorbable and that even slightest alterations in the molecular structure of cholesterol, such as stereoisomerism, prevent absorption of this very easily absorbable substance. This shows that a high specificity exists in the intestinal wall with respect to the absorption of sterols and that this is dependent upon the chemical structure.

There is no doubt that irradiated ergosterol (vitamin D) is

absorbable, but there was no proof whether the unirradiated form, which is found in all animal materials in smallest amounts, is absorbable at all, or is formed in the body itself. As the detection of ergosterol with biological or spectrophotometric methods is about 1000 times as sensitive as the methods for detection of other sterols, even a very small absorption could not escape detection.

The following experiments were carried out. Mice, rats, and rabbits were fed over a long period with very carefully purified ergosterol, the total sterols of the bodies of the killed animals were isolated and the ergosterol content of these sterols estimated with the spectrophotometric method (kindly carried out by Professor Windaus of Goettingen). There was no difference in the amount of ergosterol in fed and non-fed animals.

As these experiments only demonstrated that ergosterol cannot be stored, absorption itself was studied by the examination of the fluid of the thoracic duct during the absorption of a mixture of cholesterol and ergosterol. Only pure cholesterol with the very small normal content of ergosterol was found.

The question whether these experiments are sufficient to lead to the assumption that ergosterol must be formed in the animal body as well as in plants will be discussed.

DETERMINATION OF VITAMIN A BY A PREVENTATIVE METHOD

By E. M. NELSON, REED WALKER, AND D. BREESE JONES

*(From the Protein and Nutrition Division, Bureau of Chemistry and Soils,
United States Department of Agriculture, Washington)*

The capacity of the albino rat to assimilate and store a large quantity of vitamin A was used in studying the relationship between vitamin A intake, growth response, and survival. Single doses of vitamin A-containing material, cod liver oil, were fed to animals on a vitamin A-deficient diet. Growth response and duration of life were found to be proportional to the dosage of cod liver oil fed. This was found to be true even though the quantity of oil ingested at one time was sufficient to prolong the life of the rat for 2 months. This technique has been applied in studying the rate of disappearance of vitamin A, when cod liver oil is mixed with a ration, and the storage of vitamin A in the body.

**THE EFFECT OF MINERAL OIL ADMINISTRATION UPON THE
NUTRITIONAL ECONOMY OF THE VITAMIN A FROM
BUTTER FAT**

BY RICHARD W. JACKSON

*(From the Laboratory of Physiological Chemistry, Yale University,
New Haven)*

The possibility that mineral oil in the alimentary tract might cause a significant diversion of vitamin A from the body's normal use has been tested. The minimum amount of butter fat necessary for optimal growth of the albino rat was determined. Then, groups of rats made deficient in vitamin A were given this marginal dose of vitamin A (butter fat as described above) *with or without* the administration of 0.5 cc. daily doses of mineral oil for 75 days. Growth, eye condition, vaginal smear, appearance, and autopsy findings were employed as indices of vitamin A deficiency. The conclusions are as follows: Mineral oil causes a considerable loss of vitamin A to the animal organism if the mineral oil is *mixed with* the butter fat prior to ingestion. However, the administration of the mineral oil *separately* from the butter fat results in only a slight diversion of vitamin A. Moderate increase of the butter fat intake above the marginal dosage appears to protect the animal from any vitamin A deficiency when the mineral oil is given separately.

**THE VITAMIN A CONTENT OF DIFFERENT GRADES OF ALFALFA
AND TIMOTHY HAYS AND OF HAYS CURED UNDER
VARIOUS CONDITIONS**

BY ARTHUR M. HARTMAN

*(From the Research Laboratories of the Bureau of Dairy Industry, United
States Department of Agriculture, Washington)*

One lot of alfalfa and two lots of timothy hay were purchased in the market and graded by the Federal Hay Inspection Service of the United States Department of Agriculture in accordance with the Official Hay Standards. Also portions of alfalfa hay from the same plot were cured (1) inside the barn in diffuse light of very low intensity; in the field (2) in cocks, (3) in windrows exposed only to sunlight, and (4) in windrows exposed to sunlight, rain, and dew. These portions were also graded and the vitamin A

contents of all the hays were determined. The method used was essentially that of Sherman and Munsell.¹

Results were obtained which indicated that the vitamin A content of ground or chopped hay decreases with increasing age.

Based on the performance of rats showing a gain in 8 weeks of 31 to 38 gm. when fed daily quantities of 50 to 1500 mg. of portions of ground hay which were as nearly as possible of the same age, the following relative potencies were obtained: U. S. No. 1 alfalfa, 100; U. S. No. 2 green alfalfa (cured in barn), 100; U. S. No. 2 alfalfa (in cocks), 100; U. S. No. 2 alfalfa (sunlight only), 33;² U. S. No. 3 alfalfa (sunlight, rain, and dew), 25; U. S. No. 1 timothy, 10; U. S. No. 3 timothy, 3.

THE SPECIFIC EFFECT OF VITAMIN B ON LACTATION, GROWTH, AND BLOOD CHEMISTRY

By BARNETT SURE, MARGARET ELIZABETH SMITH, M. C. KIK,
AND DOROTHY J. WALKER

(From the Departments of Agricultural Chemistry and Home Economics,
University of Arkansas, Fayetteville)

In this study the paired feeding method of experimentation was adopted. One animal is allowed a ration deficient in vitamin B and a litter mate is given the same amount of food and water and the same diet containing an abundance of vitamin B. With the plane of nutrition being kept constant, it became apparent that vitamin B plays a specific rôle (which is designated as the *per se* effect) on metabolism.

Uncomplicated Vitamin B—On a dietary regimen deficient in vitamin B, lactating rats manifest a record of 70 to 100 per cent infant mortality in the course of 7 to 14 days. On the same plane of nutrition and on the same diet containing an abundance of vitamin B, mother rats rear their entire litters of six during that period but at a subnormal rate. In weaned rats the increase in growth due to the *specific* vitamin B effect is 40 to 60 per cent. Vitamin B *per se* shows a slight increase in the non-protein nitrogen of the blood, as indicated by results of work completed to date.

Vitamin B Complex—The *specific effect* of the vitamin B complex

¹ Sherman, H. C., and Munsell, H. E., *J. Am. Chem. Soc.*, **47**, 1639 (1925).

² 27 gm. gain in 8 weeks.

shows an increase in growth of 50 to 100 per cent. Vitamin B complex *per se* produces a slight increase in cholesterol, but marked increase in lecithins, fatty acids, and iodine number of the blood.

VITAMIN D AND CALCIUM CONSERVATION IN THE ADULT RAT

By S. W. F. KLETZIEN, B. H. THOMAS, V. M. TEMPLIN, AND
H. STEENBOCK

(*From the Department of Agricultural Chemistry, University of Wisconsin,
Madison*)

Experiments were conducted to determine the effect of vitamin D in the adult rat, with males for control and maintenance, and females for control, maintenance, reproduction, and lactation studies. The rations used were our rachitogenic Ration 2965, both irradiated and non-irradiated, and our stock ration supplemented with whole milk or whole milk powder, and with dried and irradiated dried yeast.

Adult male rats on Ration 2965 for 6 months showed a percentage reduction of femur ash from 3 to 2 times as great as on the same ration irradiated using as controls the femur ash content of animals on the stock ration or its modifications which had been taken for analysis at the initiation and termination of the experiments respectively.

Adult female rats maintained on Ration 2965 for 6 months showed a percentage reduction in femur ash which was 4 to 5 times greater than with females kept on the irradiated Ration 2965 on the basis of the femur ash of controls on the stock ration at the initiation of the experiment.

Adult female rats which were bred and which nursed litters for 3 weeks on our stock ration modified by additions of skim milk powder, additional butter fat, dried yeast, and supplemented during lactation with irradiated yeast were apparently unable to conserve a larger percentage of body calcium than comparable females without additional vitamin D. All lactating females showed losses of body calcium as compared with unbred controls.

Two successive pregnancies uncomplicated by lactation led to no apparent increase in the store of body calcium as compared with the reserves of virgin controls on the same dietetic régime.

The calcium content of young at 21 days, from females receiving additional vitamin D and those receiving none, was the same.

THE ACID-BASE EQUILIBRIUM OF RATS IN RICKETS AND TETANY

By ALFRED T. SHOHL, HELEN BENNETT BROWN, CATHERINE S. ROSE, DONALDA N. SMITH, AND FLORENCE COZAD

(From the Babies' and Children's Hospital and the Department of Pediatrics, School of Medicine, Western Reserve University, Cleveland)

Rats were made ricketic with the alkaline, high-calcium, low-phosphorus diet of Steenbock and Black, Ration 2965. The tetany produced when such animals were fasted, given phosphates—acid, neutral, or alkaline—in amounts such as are contained in normal diets, was evidenced by tremors and convulsions, carpopedal spasms, low blood serum calcium, and neuromuscular response to diminished galvanic current. For normal controls animals were fed a normal diet containing a comparable amount of alkali. For “protected” controls animals were given the ricketogenic diet plus an adequate amount of cod liver oil.

Investigation of the acid-base equilibrium of the blood serum was made upon blood obtained without contact with air. Compared to the controls, the animals with rickets showed a high normal bicarbonate content and pH and the animals with tetany a moderately diminished bicarbonate and pH. The total base of the latter group was slightly reduced.

EFFECT ON THE VITAMIN D CONTENT OF MILK OF FEEDING IRRADIATED ERGOSTEROL TO COWS

By W. E. KRAUSS AND R. M. BETHKE

(From the Nutrition Laboratory, Department of Dairy Husbandry and the Department of Animal Industry, Ohio Agricultural Experiment Station, Wooster)

Two Holstein cows in the same stage of lactation, kept under winter feeding conditions, and consuming a good dairy ration, were fed various amounts of irradiated ergosterol dissolved in Mazola, over 3 week periods. The vitamin D content of representative samples of butter fat collected during each period was determined biologically, by both the curative and prophylactic procedures, and compared with the vitamin D content of butter fat from the same cows when an equal volume of Mazola was given. The vitamin D content of the butter fat increased as the number of rat units of vitamin D fed increased—from 0.18 Steen-

bock units per gm. during the control period to 2.5 units per gm. when 200,000 rat units of vitamin D were fed—a 14-fold increase. This relationship was confirmed by bone ash values obtained in prophylactic trials.

BERYLLIUM RICKETS

By H. D. BRANION, B. L. GUYATT, AND H. D. KAY

*(From the Department of Biochemistry, University of Toronto,
Toronto, Canada)*

By replacing the calcium carbonate in Steenbock's rachitogenic Ration 2965 with an equivalent amount of beryllium carbonate, bone lesions which are similar to rickets may easily be produced in young rats. Quantities of beryllium as low as 1 per cent of the diet will bring about these effects. Both radiographic pictures and histological sections reveal an almost complete failure of calcium deposition immediately proximal to the epiphyseal disc of the long bones. At the same time the inorganic phosphate content of the blood plasma is extremely low, being usually below 1.3, and in a few cases down to 0.6 mg. of P per 100 cc. of plasma. On chemical analysis the bones show a very low mineral content and no detectable quantities of beryllium (*i.e.* less than 1 part in 2000) in the bone ash.

The onset of this type of rickets is preventable neither by administration of cod liver oil nor of irradiated ergosterol, both in generous quantities, nor by irradiation with ultra-violet light.

THE EFFECTS OF HIGH DOSES OF ALUMINUM AND IRON ON PHOSPHORUS METABOLISM

By GERALD J. COX, MARY L. DODDS, HELEN B. WIGMAN, AND
F. J. MURPHY

*(From the Utensil Fellowship, Mellon Institute of Industrial
Research, Pittsburgh)*

When guinea pigs are fed a ration containing soluble aluminum salts *in excess* of the total phosphorus of the diet, they show a marked lowering of the inorganic phosphorus of the blood to as low as 15 per cent of the normal. The ash, calcium, and phosphorus of the bones are reduced to 70 per cent of the normal in a 12 weeks period. Ferric salts produce a similar but less marked

effect. Addition of monosodium phosphate equivalent to the aluminum or iron entirely prevents the occurrence of the symptoms.

If rabbits are fed the same rations the inorganic blood phosphorus drops rapidly if the iron or aluminum of the diet exceeds its phosphorus content and after 9 days excretion of phosphorus in the urine practically ceases.

The effects produced are due to precipitation of alimentary phosphorus as the ferric and aluminum phosphates.

The phosphorus of the diet employed was equivalent to 1350 parts per million of aluminum and 2800 parts per million of iron. The amounts of aluminum and iron added were 1400 and 2900 parts per million, respectively. The quantities of these elements are many times greater than those encountered in foods of any source.

THE LACK OF RELATIONSHIP BETWEEN THE CALCIUM, PROTEIN, AND INORGANIC PHOSPHORUS OF THE SERUM OF NON- NEPHRITIC CHILDREN

BY GENEVIEVE STEARNS AND G. CLINTON KNOWLTON

(From the Department of Pediatrics, College of Medicine, State University of Iowa, Iowa City)

Several investigators have demonstrated that in nephritic children and adults, the serum calcium varies directly with the protein, and, within limits, inversely with the serum inorganic phosphorus. Peters and Eiserson³ gave to these observations a more general application, although 85 per cent of their values were from nephritic patients.

In the present series, 82 sera from children varying in age from birth to 16 years were analyzed. In no case was there evidence of renal or cardiac disease, nor any reason to believe there was a primary disturbance of the calcium metabolism or of the acid-base equilibrium. The necessary variations in level of serum protein and phosphorus were obtained through the use of the varying age groups. The protein values ranged from 4 to 8 per cent; the inorganic phosphorus, from 3.5 to 7.5 mg. per cent. The sera were grouped first according to phosphorus content, then, within the

³ Peters, J. P., and Eiserson, L., *J. Biol. Chem.*, **84**, 155 (1929).

"constant phosphorus" levels, according to increasing protein content. No significant variation in the level of serum calcium was observed with changes in either serum protein or phosphorus, although the differences in protein level were sufficient to cause, in nephritic persons, alterations in serum calcium of approximately 1 mg. per cent.³

**THE RELATION BETWEEN THE SERUM CALCIUM, PROTEIN, AND
INORGANIC PHOSPHORUS IN EARLY AND LATE PREG-
NANCY, DURING PARTURITION AND THE PUERPER-
IUM, AND IN NON-PREGNANT WOMEN**

BY W. FRED OBERST AND E. D. PLASS

*(From the Department of Obstetrics and Gynecology, College of Medicine,
State University of Iowa, Iowa City)*

The general observations of Peters and Eiserson, and others, that serum calcium varies directly with the protein and inversely with serum inorganic phosphorus, have been extended to the study of early and late pregnancy, parturition and the puerperium, as compared with normal non-pregnant women.

The sera from 98 individuals were examined. All renal or cardiac disturbances were excluded.

The sera were grouped according to protein content. No definite change in the level of serum calcium was observed with changes of protein or phosphorus. The protein content of sera in early pregnancy was somewhat lower than in the non-pregnant individual, but there was no significant change in the calcium. In late pregnancy and at parturition both the calcium and protein values were lower than in the normal non-pregnant woman, but the calcium values did not decrease directly with the protein. The protein returned to normal by the 7th to the 9th day post-partum, but the calcium remained low as at delivery, while the phosphorus values were above that of the normal.

The average index number calculated from the equation of Peters and Eiserson

$$\text{Ca} + 0.255 \text{ phosphorus} - 0.556 \text{ protein} = K$$

averaged 7.4 in the non-pregnant women. In early pregnancy the value obtained for K was 7.6, in late pregnancy 6.8, at parturition 7.4, and 7 to 9 days after delivery 7.0.

The data indicate clearly that in pregnant and non-pregnant individuals, who have no primary disturbance of the calcium metabolism or definite renal insufficiency, changes in the serum protein between 4.8 and 8.0 per cent and in the serum inorganic phosphorus between 2.8 and 5.1 mg. per cent are not necessarily accompanied by a direct change in the level of serum calcium.

**THE COMPARATIVE EFFECTIVENESS, IN A COW'S RATION, OF
SUPPLEMENTS OF PHOSPHORUS IN THE FORM OF ORTHO-
PHOSPHORIC ACID, MONO-, DI-, AND TRI-
SODIUM PHOSPHATE**

BY WILLIAM A. TURNER, E. A. KANE, AND W. S. HALE

*(From the Bureau of Dairying of the United States Department of Agriculture,
Beltsville, Maryland)*

Calcium and phosphorus balances were determined weekly for 25 consecutive weeks with three Holstein cows giving 17, 20, and 25 kilos of milk daily. All cows were pregnant and in the 4th month of lactation when the experiment began. Two of the cows aborted early in the experiment and were bred again. At the end of the experiment they had advanced 7, 5, and $1\frac{1}{2}$ months in pregnancy and the milk yields had dropped to 10, 12, and 23 kilos. Cows received more than their energy requirements according to the Savage standard and gained in weight with the exception of one cow which was unable to consume sufficient food during the hot weather and lost some weight. After the first 8 weeks the cows received 10 minutes of exercise daily.

A basal ration of Grade I alfalfa hay and grain mixture (whole yellow corn meal 40, wheat bran 30, soy bean meal 20, linseed meal 10, NaCl 1) was varied by substituting Grade I timothy hay for one-half of the alfalfa hay and by adding phosphorus supplements in the form of H_3PO_4 , NaH_2PO_4 , Na_2HPO_4 , and Na_3PO_4 so as to give a Ca:P ratio of 1.0:1.5.

Calcium and phosphorus balances were generally negative on the basal ration but much more negative when timothy hay was fed. H_3PO_4 did not prove a good source of supplementary phosphorus, balances being poorer than on the basal ration but the sodium salts, particularly the mono- and disodium phosphates, produced strongly positive calcium and phosphorus balances as well as improved assimilation and percentage assimilation.

**A STUDY ON THE PHOSPHORUS DISTRIBUTION IN RAT STRIATED
MUSCLE AS INFLUENCED BY AGE, DIET, AND IRRADIATED
ERGOSTEROL**

By **VERSA V. COLE**

*(From the Department of Physiological Chemistry and Pharmacology, the
University of Chicago, Chicago)*

The acid-soluble, acid-insoluble, labile plus inorganic, and labile phosphorus fractions were determined on the gastrocnemii muscles of albino rats at different ages, on normal stock diets, on the McCollum Diet 3143, and on the rachitic diet plus irradiated ergosterol. The rats studied ranged in age from 21 days, the weaning age, to 190 days of age. The muscles of the albino rat at weaning contain a lower percentage of labile, labile plus inorganic, and acid-soluble phosphorus, and a lower percentage of acid-insoluble phosphorus than at any other age investigated either on the stock or rachitic diets. Within 2 weeks after weaning the labile phosphorus has reached its maximum and constant value. With increased age there is an increase in the labile plus inorganic phosphorus content of the gastrocnemius muscle up to about 3 months of age and then a fall to a lower level. The labile plus inorganic phosphorus is lower in the stock animals than in the rachitic animals after about 8 weeks of age. The acid-soluble phosphorus rises to about the same height in both groups but this height is reached in 2 weeks after weaning in the stock animals and 10 weeks after weaning in the rachitic animals. The final level of the stock animals was below that of the rachitic animals. The muscles from animals kept on the stock diet gradually show a lowering in the per cent of acid-insoluble phosphorus with increase in age until a fairly constant value is reached at about 3 months of age. If kept on the rachitic diet, this fall in acid-insoluble phosphorus is more rapid and to a lower level but it then rises until at 4 months of age the level is the same as in the stock animals. The administration of irradiated ergosterol added to the rickets-producing diet in amounts up to 20,000 times the therapeutic dose does not influence muscle phosphorus distribution.

PLASMA PHOSPHATASE IN EXPERIMENTAL HYPERPARATHYROIDISM

BY AARON BODANSKY AND HENRY L. JAFFE

(From the Laboratory Division, Hospital for Joint Diseases, New York)

The plasma phosphatase of adult and young dogs was unchanged for 8 hours after the injection of single doses of parathormone. When the doses were large enough to cause hypercalcemia 24 hours after the injection, an appreciable increase of phosphatase was found at that time.

Guinea pigs were injected with large single doses of parathormone. 1 and 2 days after the injection the plasma phosphatase was considerably *lower* than in normal controls. Thereafter the plasma phosphatase returned to normal.

Guinea pigs were injected with gradually increasing doses of parathormone long enough to produce severe bone lesions. 1 day after the treatment was discontinued the phosphatase was perhaps somewhat lower than normal. At the 2, 3, and 5 day intervals the phosphatase was considerably higher than normal. On the 7th day it had returned to normal.

The plasma phosphatase is apparently influenced by parathormone secondarily, and not directly.

THE TECHNIQUE AND INTERPRETATION OF BLOOD PHOSPHATE CURVES AFTER GLUCOSE ADMINISTRATION

BY D. ROY McCULLAGH

(From the Department of Biochemical Research, Cleveland Clinic, Cleveland)

The changes in blood inorganic phosphate after the administration of 100 gm. of glucose to normal adults have been studied in 60 individuals and found to be regular. The greatest depression of phosphate occurs 2 to 3 hours after the administration of glucose and is always after the high point in the blood sugar curve. The average lowering of blood inorganic phosphate is 0.6 mg. per 100 cc.

Similar studies were made in a group of 125 patients with disease of the pancreas, thyroid, parathyroid, pituitary, or adrenals. As suggested by earlier work the phosphate changes are of diagnostic value in these cases. This value, however, is very limited since the changes are not particularly regular or marked. Many cases

of hyperthyroidism show phosphate changes very similar to those found in diabetes when such a diagnosis is clinically impossible.

Phosphatase action rapidly changes the amount of inorganic phosphate in blood *in vitro*. This change is very irregular being dependent on temperature, hydrogen ion concentration, and hence, also glycolysis. These alterations might take place between the time the blood is drawn in the wards and the commencement of analysis. A series of fifteen curves was made in which estimations were made immediately after drawing the blood and also 2 or 3 hours later. Although there was always a definite change in the amount of blood phosphate, in thirteen cases out of the fifteen, the essential shape of the curves was the same.

The statement that the rate of phosphate excretion in urine is temporarily diminished after the administration of glucose is confirmed.

PRESERVATIVE FOR SMALL BLOOD SAMPLES SENT THROUGH THE MAILS

BY ANTON R. ROSE AND FRED SCHATTNER

(From the Laboratory of The Prudential Insurance Company of America, Newark)

The chlorobenzene, sodium fluoride preserved samples received by mail since 1924 have been entirely satisfactory where single samples have been sent in for sugar determination, but with increasing demand for sugar tolerance tests requiring several samples at stated time intervals it is deemed desirable to take smaller samples. A procedure more suitable for minute blood volumes was therefore devised. Blood samples of 0.1 and 0.2 cc. volume are sufficiently dehydrated by anhydrous sodium sulfate to check its glucolytic changes. 15 gm. of crystals ($\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$) and 200 mg. of sodium fluoride are heated in a glass dish. The water of crystals dissolves both salts and is evaporated and the residue gently ignited. The powdered desiccant is measured into glass tubes 30×15 mm. with 2 mm. walls. The blood from a finger prick is measured accurately and transferred carefully to the powder and stirred with a rod permanently attached to the tight fitting rubber stopper of the sample tube. In the laboratory a deproteinizing solution is mixed with the sample and the blood

sugar determined in the filtrate. We use hydroferricyanic acid as a protein precipitant. Such samples have been set aside for from a few days to more than 3 months over a period of $1\frac{1}{2}$ years. In one-third of the cases the final sugar values checked the fresh sample by 2 per cent and in half of the cases to within 5 per cent. The half dozen failures in the earlier period may be attributed to improper transfer or mixing.

CHANGES OF *l*-ARABINOSE AND *d*-XYLOSE UNDER THE INFLUENCE OF DILUTE ALKALI

By W. C. AUSTIN, C. J. SMALLEY, AND M. I. SANKSTONE

(From the Department of Physiological Chemistry, Loyola University Medical School, Chicago)

l-Arabinose and *d*-xylose were prepared from mesquite gum and corn-cobs respectively. On theoretical grounds, and in the light of previous experience with other aldoses, it would be expected that *l*-arabinose, dissolved in a dilute alkali, would undergo partial rearrangement into *l*-ribose and *l*-araboketose. Similar treatment of *d*-xylose should give some *d*-xylose and *d*-xyloketose.

Studies were undertaken to determine the degree of changes of these two aldoses in a solution of saturated calcium hydroxide. The *l*-arabinose was dissolved in clear filtered calcium hydroxide solution, previously saturated with the base at 35°, to make the solution 0.5 M with respect to the sugar. A second solution of *d*-xylose was made in similar manner. Changes of the following values on these two solutions, kept at 35 to 40°, were studied: pH, total aldoses, total reducing sugars, specific rotation.

The results of the estimations on the *l*-arabinose system indicate that a maximum of 3 per cent of the aldose may have been converted to the ketose, and that as much as 37 per cent of the *l*-arabinose may have been converted to *l*-ribose.

The changes of the *d*-xylose system indicate that a maximum of 3.5 per cent of this aldose may have been converted to the corresponding ketose, and that as much as 24 per cent of the *d*-xylose may have been converted to *d*-lyxose.

The work is being continued in attempts to isolate the *l*-ribose, *d*-lyxose, and unknown ketoses.

THE RELATION OF GLUCOSE TO WATER RETENTION

BY J. F. McCLENDON

(From the Laboratory of Physiological Chemistry, University of Minnesota, Minneapolis)

From a study of clinical literature, low carbohydrate diets have been given in diabetes, epilepsy, fermentative dyspepsia, irritative colitis, and migraine, and in some cases of eczema. McQuarrie has shown that the low carbohydrate diet in epilepsy causes dehydration, but no biochemical justifications for the other diets (except in diabetes) have been given. Therefore, a glucose endurance test of 50 gm. per hour for 8 hours was devised to test whether there was a lessened degree of glucose tolerance in patients receiving these other diets. A normal person showed a rapid rise, then a slow fall in blood sugar which, at the end of 5 hours, was 30 per cent above normal and then continued to decline slowly. There was also an increase in blood volume (and body weight) up to this point and then a *decline*. The patient showed the same increase in blood sugar at the end of 5 hours but that was followed by a *rise*, and the same was true of the blood volume (and body weight). With insulin the patient showed a fall in blood sugar, blood volume, and body weight. The increase in body weight of the patient with the glucose test can all be accounted for by the increase in blood volume. Since, however, the glucose caused a weeping eczema in the patient, it is supposed there was a redistribution of water in the body. Water was probably lost from the muscles and carried to the skin. This was not true of the normal. A mouse fed on amino acids instead of protein for a month, was fed 24 hours on glucose with the result of marked edema of the skin.

ACTIVE GLUCOSE. THE RATE OF ITS FORMATION AS A FACTOR IN DETERMINING THE SPEED OF GLUCOSE OXIDATION

BY JOHN M. ORT AND MARTIN H. ROEPKE

(From the Division of Physics and Biophysical Research, The Mayo Foundation, Rochester, Minnesota)

At the last meeting, data were presented to show that only a very minute amount, roughly not to exceed 1 part in a quarter million, of a reductant sufficiently active to combine readily with

oxygen was present in glucose solutions from pH 7 to 10. We are now able to show that when this reductant is removed it is replaced by more at a speed much greater than might be expected from the amount that can accumulate at any one time. This has been done by two independent methods: (1) a potentiometric method with potassium ferricyanide, and (2) a colorimetric method using indophenol in the absence of iron, platinum, or any known catalyst. These data for the velocity of the formation of the active reductant show that the amount found to accumulate under the conditions studied was replaced at least within a minute when it is destroyed by an oxidant. Apparently the accumulated amount is kept small by a rapid reverse reaction in equilibrium with the first. Hence even if the concentration of the active glucose be vanishingly small in the dilute biological fluids, a fairly rapid oxidation with this mechanism as the first step can yet be possible since a rapid replacement of reductant makes the supply available for oxidation per unit of time comparatively large.

FURTHER OBSERVATION ON ACTION OF DUODENAL EXTRACT ON BLOOD SUGAR

By N. B. LAUGHTON, A. BRUCE MACALLUM, I. M. RABINOWITCH,
AND E. M. WATSON

*(From the Departments of Physiology, Biochemistry, and Clinical Chemistry,
University of Western Ontario Medical School, London, Ontario,
Canada, and the Clinical Laboratory of the Montreal General
Hospital, Montreal, Canada)*

The active principle reported previously⁴ has been separated in the form of a solid mixture representing about one four-hundredth of the original weight of the moist mucosa and practically free from the major portion of non-active material of the original preparations.

There is no effect on the blood sugar values of the totally depancreatized dog but in partially depancreatized dogs the blood sugar will rise from 100 mg. to 400 mg. in 1 hour and remain at this figure for at least 4 hours after oral administration of sugar. After oral administration of the duodenal principle prior to the administration of sugar by mouth, the blood sugar rises to about

⁴ Laughton, N. B., and Macallum, A. B., *Canad. Med. Assn. J.*, **23**, 348 (1930).

300 within 1 hour but there is a steady fall and the normal values are reached at the end of 2 hours.

In normal rabbits the duodenal preparation is introduced subcutaneously $\frac{1}{2}$ hour before intravenous sugar administration and the blood sugar reaches its peak in 10 minutes and returns to normal in 15 to 25 minutes while control rabbits with the sugar alone require 1 to 2 hours for the blood sugar to reach the initial level. After a series of repeated injections the effects persisted for a period of several weeks after the treatment had ceased.

The duodenal principle has no hormone effect on the pancreatic secretion in dogs whereas control experiments with fresh acid preparations of duodenal mucosa have an immediate effect, producing a profuse flow of pancreatic juice.

The duodenal preparation used in the above experiments has no effect on blood pressure or respiratory rate in dogs and rabbits when given in large quantities by the intravenous route.

Desiccated defatted mucosa has been prepared in the form of a light flocculent powder and contains the active principle and both the desiccated material and the active concentrate have been in clinical use for some time.

Clinical experiences with the extract appear to parallel results of animal experiments. Just as no effects whatever have been observed in the totally depancreatized, compared with the normal or partially depancreatized, animal, so the effects observed were more marked in mild than in severe human diabetes; in insulin-treated cases, the effects were either nil or of a temporary nature only; in non-insulin treated cases, lowering of blood sugar appeared to be related to the mildness of the diabetes. In cases on the border-line, with respect to the use of insulin, it has been observed repeatedly that, though the blood sugar was lowered, the effects were temporary, hyperglycemia reappearing after short periods. No patients have been observed for any sufficient length of time to warrant any conclusions as to the possible therapeutic value of this extract.

Differing from the effects caused in animals, the effects of the extract on blood sugar in the human individual are not immediate. Blood sugar-time curves obtained following glucose ingestion and extended over a period of 3 hours showed a depression of plasma phosphate alone; there were no alterations either

of blood sugar or respiratory quotients. It would, therefore, appear, that shortly after glucose ingestion, the only effect of the extract is combination between glucose and phosphorus—the apparently necessary preliminary to utilization.

The authors acknowledge the assistance and cooperation of Doctor D. M. Lawrason.

THE NATURE OF BLOOD SUGAR

By MICHAEL SOMOGYI

(From the Laboratory of the Jewish Hospital of St. Louis, St. Louis)

Examination of the optical activity of blood sugar is rather difficult due mainly to two factors. First, the concentration of sugar in protein-free blood extracts is very low, and second, the extracts contain other optically active substances besides sugar. Some constituents of the extracts also reduce copper in alkaline solution, a circumstance that renders the correlation of reduction values with optical activity utterly impossible. The writer employed a technique of deproteinization which yields filtrates of the original concentration of blood or plasma, and does not contain measurable amounts of either optically active or of reducing substances other than sugar.

The deproteinization is performed in two stages. First, CuSO_4 in substance is added to the blood or plasma, followed by neutralization with powdered BaCO_3 . The protein-free filtrate thus obtained is then shaken with finely powdered HgSO_4 and again neutralized with BaCO_3 in order to remove almost completely the non-protein nitrogenous substances (West's technique). The Hg and Cu still retained in solution are removed by H_2S and the latter is blown off by a stream of nitrogen. The resultant colorless fluid contains the blood sugar in the original concentration, so that it is possible to make accurate polarimetric determinations. In a 400 mm. tube the polarimetric measurements in the present experiments amounted to $0.2\text{--}0.7^\circ$. The amount of the sugar, as calculated from its copper reduction value, was correlated with the optical rotation, and the relation between these two quantities was found to be the same as in the case of α - β -glucose. In addition the rate of oxidation with copper solutions of different

degrees of alkalinity, and finally the rate of fermentation and the amount of CO_2 produced in fermentation were determined in comparative experiments with blood filtrates and solutions of ordinary glucose, and the behavior of blood sugar in all of these relations was found to be identical with that of ordinary glucose.

THE RELATIVE ANTIKETOGENIC VALUE OF GLUCOSE AND GALACTOSE

By HARRY J. DEUEL, JR., MARGARET GULICK, AND J. S. BUTTS
(From the Department of Biochemistry, University of Southern California
School of Medicine, Los Angeles)

It is generally assumed that all of the digestible carbohydrates possess the same antiketogenic value. Goldblatt,⁵ however, has stated that galactose, mannose, lactose, and glycerol are inert while glucose, sucrose, fructose, and maltose alone are effective in this respect. These results were seemingly at variance with some earlier work of Deuel and Chambers⁶ in which a nitrogen sparing action (which parallels the antiketogenic effect in phlorhizinized dogs) was noted after the administration of galactose and lactose to such animals.

The present experiments have been carried out on normal men and women in whom an acidosis was produced either by a prolonged protein-fat diet or by a period of fasting. In all three experiments on three subjects taking a protein-fat diet, the antiketogenic effect produced by a dose of galactose from 25 to 75 gm. was much more marked than that occasioned by a similar amount of glucose. In two cases the administration of 25 gm. of glucose was not followed by any detectable drop in ketone body excretion while a definite decrease was noted after the ingestion of the same quantity of galactose. In the fasting experiments which usually lasted for a week, the sugar was administered on the 5th day after the acetone excretion had reached a fairly constant level. In two out of three cases, the galactose showed a markedly greater potency than did the glucose while in the third case only slightly better results were obtained with the former. The urinary nitrogen which was decreased the 1st day of the fast,

⁵ Goldblatt, M. W., *Biochem. J.*, **19**, 948 (1925).

⁶ Deuel, H. J., Jr., and Chambers, W. H., *J. Biol. Chem.*, **65**, 7 (1925).

rose on the 2nd day presumably due to the exhaustion of the glycogen supply. The decrease in urinary nitrogen was marked after the ingestion of the sugars and was parallel to the drop in the acetone. With galactose, the lowered acetone output lasted until the 3rd day, while in most cases with glucose, the antiketogenic effect was complete within 2 days. On one subject in which extremely consistent results were obtained on four consecutive fasts, the galactose exerted a sparing effect approximately 80 per cent greater than did the glucose.

REDUCING SUBSTANCES IN URINE

By EDWARD S. WEST

*(From the Department of Biological Chemistry, Washington University
School of Medicine, St. Louis)*

A study of fermentable and non-fermentable reducing substances in urine has been made based upon estimation in HgSO_4 - BaCO_3 filtrates, with use of washed yeast and a sensitive Shaffer-Hartmann sugar reagent.

The average daily excretion of fermentable sugar was 142 mg. for 56 normal adults on unrestricted diets. The maximum output was 416 mg. and the minimum 44 mg. The average for non-fermentable reducing substances was 395 mg. (calculated as glucose), with a maximum of 562 mg. and a minimum of 273 mg. in 58 normal adults.

Hospital patients with kidney impairment showed retention of non-fermentable reducing substances.

The excretion of non-fermentable reducing substances was found to be especially related to the ingestion of dried fruits, apples, honey, dark table syrup, and caramelized carbohydrate material in general. High protein diets caused no increase. In experiments on the dog, the output continued throughout prolonged fasting at a lower but rather constant level. Constipation caused a marked increase.

Fermentable sugar excretion in normal persons was found to be apparently related to the condition of activity of the pancreas or general carbohydrate metabolism. It continued throughout prolonged fasting in the dog.

THE KETOSE OF NORMAL URINE

BY MARK R. EVERETT AND FAY SHEPPARD

(From the Department of Biochemistry and Pharmacology, University of Oklahoma Medical School, Oklahoma City)

The response of the sugar of normal night urine to bromine⁷ and to alkaline iodine is that of a ketose. We have prepared concentrated syrups of this sugar, which contain only traces of nitrogenous substances, chlorides, etc. The sugar in these preparations is only a small fraction of the original, but it resembles the major portion in its properties. A non-reducing, organic acid is present as an impurity and has been separated as its insoluble silver salt.

These syrups are optically inactive or slightly dextrorotatory. Diluted portions give an intense response to Tashiro's test,⁸ which is much more specific in urine than Selivanoff's test. The uroketose does not resemble *d*-xyloketose. The syrups give orange colors with Bial's reagent, to be replaced by green, only after momentary boiling. The naphthoresorcinol test gives misleading colors with normal urine, since they are not diminished by previous bromination of the urine. The uroketose syrups give brown colorations, with no trace of purple.

The uroketose is precipitated completely by Goulard's extract and sodium hydroxide, but less easily by ammoniacal lead acetate or barium hydroxide. A crystalline phenylhydrazine derivative has been prepared from the nitrogen-free syrups. Its melting point is higher than that of isomaltosazone, which Baisch⁹ and others claimed to have found.

THE RELATION OF INSULIN TO LIVER GLYCOGEN

BY R. C. BODO AND ISAAC NEUWIRTH

(From the Department of Pharmacology, New York University, New York)

The work which is reported here was done on dogs, which preliminary to the actual experiment were fed on a high carbohydrate diet. Morphine and chloralose were used for anesthesia. Sam-

⁷ Everett, M. R., and Sheppard, F., *J. Biol. Chem.*, **87**, p. xxxv (1930).

⁸ Tashiro, S., and Tietz, E. B., *J. Biol. Chem.*, **87**, 307 (1930).

⁹ Baisch, K., *Z. physiol. Chem.*, **18**, 193 (1894).

ples of liver (about 5 gm.) were cut out at hourly intervals, each time from a different lobe, bleeding being stopped by mass ligation. Blood samples were taken for sugar analysis at times corresponding to the liver section. Where sugar was infused, a slow continuous infusion was made into the portal vein, hepatic artery, or femoral vein. Where insulin, commercial or crystalline, was given, this was injected intravenously or subcutaneously, either in single doses or infused at a slow constant rate into the portal or jugular vein. The experiment usually lasted 5 hours, the animal's condition as shown by blood pressure remaining satisfactory during this period. The results of our work can be given briefly as follows:

In a normal dog under the experimental conditions described, with no sugar infusion, there occurs a steady decrease in liver glycogen during the 5 hour period, the blood sugar falling slightly. On the other hand, if sugar is infused, a rise in liver glycogen occurs. These experiments establish that the technique employed does not affect materially the normal function of the liver in regard to glycogen.

In a normal dog with sugar infusion and relatively large amount of insulin given in single injections, the liver glycogen falls. With continuous slow infusion of insulin in small but effective amounts (4 units per hour) no appreciable change occurred. It follows from this that insulin in large dosage causes a breakdown of glycogen; in small doses it prevents a storage and as no hypoglycemia was present in our experiment, this breakdown is due to a direct liver action.

In a third group of experiments, the pancreas was removed at the time of the experiment. When no sugar infusion was made, a great fall in liver glycogen occurred. With sugar infusion a fall also occurred. With sugar and insulin infusion, the greatest fall resulted.

Owing to the possibility of the operative procedure, through trauma, being partly responsible for the rapid breakdown, a final group of experiments was carried out in which the pancreas was removed 48 hours or more before the actual experiment. During this preparatory period one dog was given insulin and sugar, another was not. In both instances, in the actual experiment during which sugar and insulin were infused, an increase in liver glycogen occurred.

In reviewing our experiments we see that in a diabetic dog insulin with sugar brings about a rise in liver glycogen, and the insulin is a necessary factor in this effect. This rise in glycogen occurs from the very beginning and is a more or less gradual one. On the other hand, in a normal dog, insulin with sugar produces a decrease in glycogen or at least prevents storage. We must assume therefore that insulin has two actions. The breakdown of glycogen is due to direct liver action, as has also been shown previously on the perfused isolated liver of normal animals. No such definite statement can be made concerning the storage action, which may be a direct one, but is equally possibly an indirect one, that is by changing the general metabolism in such a manner that the liver cells regain their normal storing function. We plan further perfusion experiments on isolated livers from diabetic dogs, which should throw light on the matter.

DOES ANY CARBON DIOXIDE IN THE BLOOD EXIST AS CARBHEMOGLOBIN?

BY WILLIAM C. STADIE AND HELEN O'BRIEN

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia)

Bohr's old hypothesis, supported by Bayliss and others, that a considerable part of the CO_2 in blood exists as a direct combination with hemoglobin called carbhemoglobin, had been completely replaced by the current notion that all CO_2 existed as physically dissolved CO_2 or as bicarbonate ions. Recently, Henriques showed that the rate of exchange of CO_2 from the blood in the lungs was far in excess of that calculated from velocity data obtained from simple aqueous solutions and he showed that in hemoglobin solutions the velocity of the reaction $\text{H}_2\text{CO}_3 \rightarrow \text{H}_2\text{O} + \text{CO}_2$ was greatly increased. This fact, since abundantly corroborated by Van Slyke and Hawkins (1930) and Dirkin and Mook (1930), led Henriques to revive the Bohr hypothesis that 30 to 60 per cent of the CO_2 of the blood was in the form of carbhemoglobin. To support this hypothesis Henriques measured the activity of the bicarbonate ion in hemoglobin solutions by the Donnan membrane method and found, as had Stadie and Hawes (1928), that $a_{\text{HCO}_3^-}$ was diminished by the hemoglobin. This he attributed to

carbhemoglobin formation, whereas Stadie (1929) concluded it was due to a simple physicochemical mechanism depressing the activity coefficient of the bicarbonate ion.

Stadie and O'Brien in this paper present two types of evidence on the problem.

1. A hemoglobin solution containing a known concentration of Na was divided into two portions. The first was titrated with HCl and the pH determined electrometrically. From the data, NaHb can be calculated at any pH. The second portion was equilibrated with CO₂ and the pH and total CO₂ were determined. The total CO₂ is

$$\text{CO}_2 = \text{H}_2\text{CO}_3 + \text{NaHCO}_3 + \text{HbCO}_2$$

also

$$\text{NaHCO}_3 = b - \text{NaHb}$$

where b is total base and HbCO₂ is hypothetical carbhemoglobin. All factors are now known except HbCO₂, which may be calculated. It was found that no appreciable amount of CO₂ was present as HbCO₂.

2. Solutions of hemoglobin containing known base (Na) were equilibrated with CO₂ in the Stadie and Sunderman (1931) freezing point apparatus and the depression (Δ) of the freezing point determined. The concentration of the bicarbonate ion can be calculated by the equation

$$C_{\text{HCO}_3^-} = \frac{\Delta}{1.86f}$$

where f = osmotic coefficient. It was found that the $C_{\text{HCO}_3^-}$ osmotically determined and the H₂CO₃ calculated from the CO₂ pressure accounted within ± 4 per cent for the total CO₂.

The conclusion from the two types of experiment is that no carbhemoglobin exists in the blood but that all CO₂ is in the form of H₂CO₃ or HCO₃⁻.

THE NITROGEN CONTENT OF BLOOD

By C. FERDINAND NELSON AND W. M. COLE

(From the Department of Biochemistry, University of Kansas, Lawrence)

The free nitrogen present in freshly drawn blood of a large number of healthy men and women, and animals (calf, ox, pig,

dog, chicken, and guinea pig) has been determined with the Van Slyke-Neill manometric blood gas apparatus for extracting and measuring the gas present. With from 2 to 5 cc. of blood collected under oil and sodium hydrosulfite, to absorb oxygen, the mean value for free nitrogen was found to be 0.96 cc. per 100 cc. of blood. For aerated blood, under similar conditions, the mean free nitrogen value was found to be 1.05 cc.

After the carrying through of the technique of Sendroy and Liu for determining carbon monoxide in blood the residual free nitrogen values found were as follows:

Unaerated blood, 2 cc. volume.....	1.30 cc.
Aerated " 2 " "	1.45 "
Unaerated " 4 " "	1.03 "
Aerated " 4 " "	1.06 "

THE ALKALINITY AND PHOSPHATE CONTENT OF THE MORNING URINE

BY ROGER S. HUBBARD, SAMUEL A. MUNFORD, JAMES TYNER,
AND CATHERINE B. ALLISON

*(From the Laboratories of the Clifton Springs Sanitarium and Clinic,
Clifton Springs, New York)*

The period of urinary alkalinity which usually follows a meal is slight or absent in patients who do not secrete hydrochloric acid in the gastric juice. This is shown by average figures of results of tests upon 86 subjects suffering from this condition contrasted with studies upon 125 controls.

The phosphate excretion in such patients was studied in forty tests upon twenty-four subjects. The results did not differ significantly from the data previously reported in the literature for normal human subjects.

In patients with achlorhydria there was no correlation between the phosphate excretion and urinary acidity, but some relationship existed between the concentration of these compounds and the reaction of the urine. It seems probable that this latter relationship depends, at least in part, upon a relationship between urine volume and reaction, which does not seem to be an essential one, but which is quite frequently present.

Experiments in which a dilute urine was excreted following the ingestion of large amounts of water showed a greater variation

in reaction than did others in which a concentrated urine was obtained. Statistical analysis of all the data available confirmed the results of these experiments. Such results are possibly due to a diminution of the amount of buffer present.

These two relationships—a slight correlation between phosphate concentration and reaction, and a greater constancy of reaction when the phosphate concentration was high than when it was low—were the only ones found in this study of the morning urine of patients with achlorhydria.

EFFECTS ON THE RAT OF DEPRIVATION OF MAGNESIUM

By E. V. McCOLLUM AND ELSA R. ORENT

(From the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore)

Rats, 35 to 45 gm. in weight, were restricted to a diet containing but 1.8 parts per million of magnesium. Within 3 days all exposed skin areas show marked vasodilatation. This condition becomes intensified from day to day to about the 10th day, when it begins to subside. During this period the animals are watchful, apprehensive of danger, and start at noises or at shadows passing over them. This hypersensitivity becomes progressively more pronounced until about the 11th day, when any disturbance may cause convulsions. The attack is preceded by agitation, accompanied by whirling movements, followed by collapse. This stage is quickly succeeded by clonic convulsions. The skin suddenly blanches and the eyes protrude. The rat makes no sound during the convulsive seizure. About 80 per cent die during the first attack. Some have survived several attacks.

Weight remains about stationary after rats are restricted to the magnesium-poor diet. They become emaciated. There is an extraordinary decalcification of the entire skeleton which is nearly complete in 90 days.

Deprivation of magnesium results in loss of hair on the ears, the under surface of the jaws and neck, and general thinning of the coat. Salivation and lacrymation are marked and there is some hemorrhage from the nose and eyes.

When rats which had survived the convulsive attack for 4 weeks had magnesium restored to the diet they gained 20 to 25 gm. in

5 days. Lacrymation and salivation ceased promptly and new growth of hair had begun. Their appearance rapidly returned to normal.

After 2 to 3 weeks of magnesium deprivation there appear, just below the gum line on the incisors, areas resembling the mottled enamel of human teeth described by McKay as resulting from some effect of the drinking water of certain localities throughout the world. About the 10th week extensive changes about the molars are seen, and on examination this is represented by what appears to be an extreme swelling of the tissues around the teeth. This swelling consists of a fibrous hypertrophy with a thin cover of oral epithelium.

These results present an extremely interesting condition brought about by magnesium starvation, which invites extensive physiological, histological, pathological, metabolic, and dental studies. These are in progress both with rats and dogs.

ISOLATION OF THE NATURAL ANTIOXIDANT OF LETTUCE

BY H. S. OLCOVICH AND H. A. MATTILL

(From the Biochemical Laboratory, State University of Iowa, Iowa City)

In order to segregate, chemically and functionally, the various substances occurring in the unsaponifiable portion of plant lipids, especially the commonly associated vitamin E and antioxidant, green lettuce leaves were carefully dried in a vacuum oven, continuously extracted for 24 hours with hot alcohol, saponified, and extracted with ether. The unsaponifiable material as obtained was fractionally crystallized from a number of organic solvents. The distribution of vitamin E in the various fractions was determined by their effect on female rats on a sterility-producing diet; the distribution of the antioxidant, by its capacity to prolong the induction period of an autoxidizable fat mixture. The crystallizable material contained a number of inactive solid alcohols and phytosterols which are being identified. Vitamin E and the antioxidant remained with the non-crystalline material but the latter could be separated by extracting a petroleum ether solution with 92 per cent methyl alcohol. The vitamin E activity remained in the petroleum ether.

A concentrate of the antioxidant could not be secured by

crystallization but was finally obtained by fractional distillation *in vacuo*. The oily fraction distilling from 160–180° at 0 mm., on cooling yielded crystals, which, when purified, proved to have marked antioxidant capacity. The compound forms colorless prisms from acetone, leaflets from chloroform, m.p. 142° (corrected), is very soluble in alcohol, ether, acetone, and dilute alkalis, moderately soluble in chloroform and benzene, and insoluble in petroleum ether and water. The crystals are monoclinic. The formula, from analyses and molecular weight determinations, is $C_{13}H_{14}O_5$. One or more phenolic groups are present. The anti-oxygenic index is 29, a value close to that of α -naphthol.

CHEMICAL INVESTIGATIONS OF THE LIPOIDS OF THE TIMOTHY BACILLUS

By MARY C. PANGBORN AND R. J. ANDERSON

(From the Department of Chemistry, Yale University, New Haven)

An investigation is being made of the lipoids of the timothy bacillus in comparison with the lipoids of the pathogenic tubercle bacilli. It has been found that the ether-soluble constituents obtained from all types of acid-fast bacteria so far studied are very similar in kind and consist of phosphatides, acetone-soluble fat, and wax. Quantitatively these components vary greatly with the different strains of bacteria.

The acetone-soluble fat and the phosphatide from the timothy bacillus have been analyzed and they have been found to be very similar in composition to corresponding fractions isolated from the tubercle bacilli. The fatty acids consist of saturated solid acids, liquid unsaturated acids, and liquid saturated fatty acids analogous to tuberculostearic acid and phthioic acid. The water-soluble constituents obtained on hydrolyzing the timothy bacillus phosphatide contain glycerophosphoric acid, mannose, and inositol.

THE OXIDATION OF α -HYDROXY FATTY ACIDS IN VITRO AND THE SHIFTING POINT OF RUPTURE IN THESE ACIDS

By EDGAR J. WITZEMANN

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison)

It has long been known that the oxidation of ammonium butyrate with hydrogen peroxide is initiated at two points, namely on

both the α - and β -carbon atoms. The α -oxidation is commonly believed to be of no importance in biological conditions. In earlier work,¹⁰ it was found that sodium α -hydroxybutyrate, upon further oxidation, may lose 2 carbon atoms, and thus in effect give rise to the same final result generally assigned to β -oxidation. It has now been found, in the oxidation of the sodium salts of lactic acid and of α -hydroxybutyric, α -hydroxyvaleric, α -hydroxycaproic, and α -hydroxyheptylic acids, that there is a shift in the point of rupture of the carbon chain in passing from lactic acid to α -hydroxyheptylic acid. In neutral solution lactic acid loses but 1 carbon atom (= 84 per cent). About half of the α -hydroxybutyric acid molecules lose 1 carbon atom, while the remainder lose 2 carbon atoms. The shift in the point of rupture continues on passing up the series, and with α -hydroxyheptylic acid no evidence of the loss of but 1 carbon atom could be obtained; valeric acid was obtained as the oxidation product and no caproic acid could be detected.

A similar shift is obtained with a given hydroxy acid by increasing the alkali present in the solution. These effects are interpreted as due to the increasing enolization of the α -keto acids formed as intermediates. These and the remaining results in hand give basis for declaring that these compounds appear to behave more like sugars than was to be expected.

THE LIPID CONTENT OF THE INTESTINAL MUCOSA*

By WARREN M. SPERRY

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Laboratory, Babies Hospital, Department of Diseases of Children,
College of Physicians and Surgeons, Columbia University,
New York)*

In previous reports desquamation of intestinal epithelium has been included among the possible sources of the endogenous lipid excretion which has been found to occur in dogs. Very few analyses of the lipids in postabsorptive intestinal epithelium or

¹⁰ Witzemann, E. J., *J. Am. Chem. Soc.*, **48**, 202, 211 (1926).

* This investigation was supported in part by a grant from the Chemical Foundation.

mucosa have been found in the literature. It was the purpose of this investigation to furnish such data and from them to estimate the maximum rôle of desquamation in lipid excretion. The dogs which were used as subjects were sacrificed at least 24 hours after the last feeding. The entire intestine was removed immediately after sacrifice, and the outer muscular layers were separated from the mucosa by turning the former back over the intestine, the mucosa being drawn out between the thumb and forefinger. The mucosa from the small intestine was divided according to length into two equal parts which were analyzed separately along with the colon by slitting them open longitudinally, washing thoroughly with water, cutting into small pieces, and extracting with hot alcohol and ether. The combined extracts were dried, taken up in ether, saponified with 20 per cent alcoholic KOH on the steam bath, and the petroleum ether-soluble, unsaponifiable, and fatty acid portions were separated and weighed.

Altogether seventeen dogs were studied. Of these six had had ileostomy performed, had been used in lipid excretion studies as described previously,¹¹ and were definitely abnormal; five could not be classed as normal for various reasons, though the abnormalities probably had no effect on the intestinal tract; and six were entirely normal. No difference in the results could be made out between these groups of animals.

The data lead to the conclusion that the relative amount of lipids (sum of unsaponifiable and fatty acid fractions) is smaller in the mucosa of the colon than of the small intestine. No consistent difference in the unsaponifiable contents of colon and small intestine could be made out. The higher fatty acid content of the small intestine thus indicated is probably due to a higher active phospholipid content.

Unsaponifiable material made up a smaller portion of mucosa lipids than of excreted lipids and therefore was the limiting factor to be considered in estimating the possible rôle played by desquamation in lipid excretion. It was calculated from average values that a wear and tear of 42.4 per cent of the entire mucosa per day would be necessary to account for the excretion, while a wear and tear of 5.4 per cent would be necessary if the dog with the

¹¹ Sperry, W. M., and Angevine, R. W., *J. Biol. Chem.*, **87**, p. xxii (1930).

highest mucosa unsaponifiable content chanced to have the lowest excretion found. Since the desquamation of epithelium, which makes up only a part of the mucosa, would have to be still greater, it is concluded that desquamation plays an insignificant rôle in lipid excretion.

THE COMPOSITION OF THE UNSATURATED FATTY ACIDS OF ANIMAL TISSUES

By H. GREGG SMITH

(From the Biochemical Laboratory, State University of Iowa, Iowa City)

Very little information is available as to the composition of the unsaturated fatty acids of animal tissues, especially as regards the location of the double bonds. This is a report of preliminary experiments on the fatty acids of beef heart muscle and liver. The unsaturated acids were separated by two methods, crystallization of the bromine derivatives and fractional distillation of the methyl esters. The method of Armstrong and Hilditch was used for the oxidation of the unsaturated acids and the results obtained up to this time indicate that the first double bond nearest the carboxyl group is in the 9:10 position. Azelaic acid is the only dibasic acid which has been separated from the oxidation products.

THE EFFECT OF INGESTED COTTONSEED OIL ON THE COMPOSITION OF BODY FAT

By N. R. ELLIS

(From the Nutrition Laboratory and Office of Swine Investigations, Animal Husbandry Division, Bureau of Animal Industry, United States Department of Agriculture, Washington)

Earlier work showed that the ingestion of cottonseed oil by hogs resulted in the formation of a firm body fat characterized by a high melting point and a high saturated acid content. In order to determine the level at which maximum firmness was produced, experiments were conducted on hogs in which the oil was added to basal rations at 4, 8, and 12 per cent levels. The hardest fats were secured on the 4 per cent level. The higher quantities resulted in increasing softness.

Analyses of the lard showed striking changes in the fatty acid distribution. Among these was a marked increase in the linolic

and the stearic acid at the expense of the oleic and palmitic acids. The maximum content of stearic acid occurred in the 4 per cent level. Rat feeding tests on the cottonseed oil showed a more pronounced softening of the body fat over that obtained in hogs. It is suggested that the changes in composition of the lard are due to the presence in cottonseed oil of one or more isomeric forms of oleic or possibly linoleic acid which are readily converted into stearic acid. The hydroxy stearic acids formed on permanganate oxidation of the oil showed the presence of at least one isomer of ordinary oleic acid. Studies of the oxidation products of the liver fat of hogs fed cottonseed oil are also reported.

ON THE FATTY ACIDS ESSENTIAL IN NUTRITION

By GEORGE O. BURR, MILDRED M. BURR, AND E. S. MILLER

(From the Department of Botany, University of Minnesota, Minneapolis)

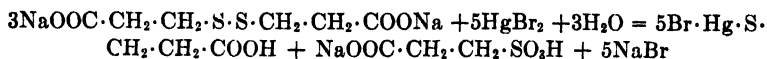
This is a continuation of work previously reported and published. Several more fatty acids and their mixtures are compared as to their effectiveness in curing rats suffering from a fat deficiency. The relation of these results to other problems is discussed.

THE SIMULTANEOUS PRODUCTION OF THIOL ACIDS (R-S-H) AND SULFONIC ACIDS (R-SO₃-H) FROM DITHIO ACIDS (R-S-S-R) BY MERCURIC BROMIDE AND OTHER METALLIC SALTS

By PAUL W. PREISLER

(From the McCarthy Neurological Foundation and the Department of Physiological Chemistry, University of Pennsylvania, School of Medicine, Philadelphia)

As a continuation of the study of the mechanism of the formation of the thiol and sulfonic acids from dithio acid, an investigation has been made of the reaction occurring with mercuric salts and salts of other metals. The quantitative analytical results indicate that with mercuric bromide in the presence of hydrobromic acid, the reaction with dithiodihydracrylic acid proceeds to the extent of at least 75 per cent according to the following equation.



The thiol acid was isolated and identified as the salt indicated in the equation and the sulfonic acid in the form of its barium salt.

With an iodimetric method as a measure of the formation of thiol acid, an investigation was made of the extent of the reaction of dithiodihydracrylic acid with salts of other metals. The reaction appears to be confined to certain metals closely related to silver in the periodic system; thus, in addition to silver (previously reported) and mercury, the reaction appears to take place also with copper, cadmium, and thallium. In the case of cupric salts, the thiol acid formed precipitates as a mono- or dicuprous derivative. Large quantities of the sulfonic acid were isolated. The reaction is complicated by the oxidizing action of the cupric salts so that the ratio of the thiol acid to the sulfonic acid produced appears to be different from that obtained in the case of silver or mercury. These reactions are being investigated further.

A discussion of this reaction as a unique example of simultaneous oxidation and reduction and also its possible significance in the sulfur metabolism of the organism was presented.

THE DECOMPOSITION OF CYSTINE PHENYLHYDANTOIN

BY MAX BERGMANN, JAMES C. ANDREWS, AND KATHLEEN
CRANDALL ANDREWS

(From the Laboratories of the Kaiser-Wilhelm Institut für Lederforschung,
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School of Medicine, University of Pennsylvania, Philadelphia)

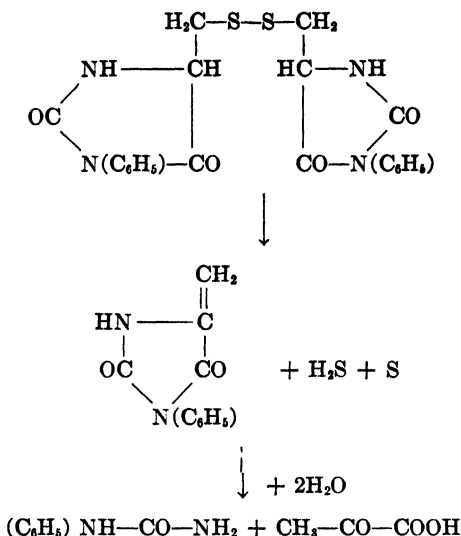
The alkaline decomposition of cystine phenylhydantoin to 3-phenyl-5-methylene hydantoin, and the further splitting of the latter to phenylurea and pyruvic acid have been studied in detail. The course of the reaction is very similar to that reported by Bergmann and Delis¹² for serine phenylhydantoin.¹³ Samples of cystine phenylhydantoin prepared by the method of Patten¹⁴ from *l*-cystine showed the theoretical composition and the melting point stated by Patten. The decomposition of solutions of the hydantoin in both acetone and pyridine was followed in the

¹² Bergmann, M., and Delis, D., *Ann. Chem.*, **458**, 76 (1927).

¹³ Delis, D., Inaugural dissertation. University of Leipsic, Leipsic (1928).

¹⁴ Patten, A. J., *Z. physiol. Chem.*, **39**, 350 (1903).

polarimeter and the end-products were isolated. The reaction proceeds as follows:



Isolation and identification of phenylurea is carried out after decomposition of the cystine phenylhydantoin in strong NaOH. Much more gentle treatment is required to produce the intermediate compound, 3-phenyl-5-methylene hydantoin, which hydrolyzes further with great ease.

The first stage in the decomposition was followed by means of the polarimeter. The hydantoin, which is very insoluble in water, was dissolved in either acetone or pyridine. In acetone the hydantoin from *l*-cystine shows in 1 per cent solution $[\alpha]_D^{20} = -176.6^\circ$ and in pyridine $[\alpha]_D^{20} = -281.9^\circ$. In both pure acetone and aqueous acetone solutions the hydantoin is permanently stable. The same is true of anhydrous pyridine, but the introduction of small amounts of water into the pyridine produces enough alkalinity to cause almost complete decomposition in 24 hours at room temperature.

Studies of the rate of decomposition caused by varying proportions of dilute alkali emphasized the great instability of the compound. The use of 1:1 molar proportion of alkali to hydantoin produced complete decomposition in 1 hour at room temperature.

There is no evidence of any formation of ammonia in the course of the decomposition.

The decomposition does not produce a constant when calculated for either a mono- or a dimolecular reaction but appears to be a mixture of the two.

The high degree of instability evidenced here corresponds to that demonstrated by Bergmann and coworkers with other ring derivatives of cystine and serine and supports the assumption that such a mechanism might be a possible mode of oxidative decomposition of these amino acids.

CHEMISTRY OF THE INTEGUMENT

I. PROTEIN BEHAVIOR IMMEDIATELY POST MORTEM

By WITHROW MORSE

(From the Manufacturing Department, Rohm and Haas Company, Incorporated, Bristol, Pennsylvania)

The cat was used. The changes observed in proteins concerned the coagulable forms, scleroproteins not participating. The albumin:globulin ratio resembled that of serum, 2:1. Globulin disappeared early under tissue protease action. The degree of such enzyme activity was correlated with the fineness with which the integument was divided, larger portions being very slowly changed. Free cystine, characteristic of hair and cuticle, appeared only late in hydrolysis, which is evidence that keratin is refractory to tissue proteases. Mucoid, identified by others in the skin of mammals having sebaceous glands distributed through the surface of the body, was not found in the cat (which has such glands restricted to the extremities). A study was made of the effects of dehydrating skin by drying or by hypertonic substances.

STUDIES ON GLUTELINS

By FRANK A. CSONKA AND D. BREESE JONES

(From the Protein and Nutrition Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington)

The proteins of buckwheat (*Fagopyrum fagopyrum*) were studied by Ritthausen and by Johns and Chernoff. The latter authors obtained a globulin representing only 20 per cent of the total

proteins present in the seed. We were able to separate by our method a glutelin from buckwheat belonging to the α -series. Van Slyke's analysis showed that this glutelin is rather high in arginine and lysine, and most likely free of proline. Its isoelectric point, pH 6.45, is characteristic of the group of proteins, glutelins.

THE MOLECULAR WEIGHT OF SERUM ALBUMIN ESTIMATED BY OSMOTIC PRESSURE MEASUREMENTS*

By NORVAL F. BURK†

(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston)

Because of the discrepancy in the estimates of the molecular weight of serum albumin made by Sørensen¹⁵ (45,000) and Adair¹⁶ (62,000) by means of osmotic pressure measurements, and by Svedberg¹⁷ by the ultra-centrifugal measurements (67,500), the osmotic pressure of this protein has been reinvestigated under more suitable experimental conditions, both in aqueous solutions and in certain mixed solvents, especially urea-water solutions.

Serum albumin is denatured by urea. Comparison of molecular weight measurements in aqueous and in urea solutions, under similar experimental conditions, makes it possible to consider whether changes in molecular weight accompany the process of denaturation of serum albumin. The recent investigations of Hopkins¹⁸ have shown that the denaturation of certain proteins by urea involves changes in the sulfur linkages in the protein molecule.

The serum albumin was crystallized, dialyzed, and electro-dialyzed. The specific conductivity varied between 1 and 3×10^{-5} reciprocal ohms. In dilute aqueous solution, buffered at

* Since submitting this abstract, the paper of Adair and Robinson has become available. Their estimate of $72,000 \pm 3000$ for the molecular weight of serum albumin is in excellent agreement with that reported.

† National Research Council Fellow in the Biological Sciences.

¹⁵ Sørensen, S. P. L., *Proteins*, The Fleischmann Company, New York (1925).

¹⁶ Adair, G. S., *Skand. Arch. Physiol.*, **49**, 76 (1926).

¹⁷ Svedberg, T., and Sjögren, B., *J. Am. Chem. Soc.*, **50**, 3318 (1928).

¹⁸ Hopkins, G., *Nature*, **126**, 328 (1930).

pH 4.8, the osmotic pressure was proportional to the protein concentration, yielding the value 75,000 for the molecular weight. The osmotic pressure in 6.66 M urea solution, buffered at its isoelectric point in this solvent, namely pH 5.8, increased with the concentration by an amount greater than proportional to the increase of protein concentration. The deviation from proportionality was corrected for by a method previously described¹⁹ and led to a value of 73,000 for the molecular weight of denatured serum albumin in urea-water mixtures.

Measurements on the osmotic pressure of serum albumin in the presence of moderate concentrations of salts confirmed those of Sørensen, and demonstrated that, when the solvent is a moderately concentrated salt solution, the ideal solution law $PV_0 = RT$ is not obeyed. Instead the relationship $P(V_0 - b) = RT$ has been found to describe the data, where V_0 is the volume of the solvent, and b a constant. A recalculation of the measurements of Sørensen, which were carried out at high protein and salt concentrations, in terms of this relation leads to a value for the molecular weight of serum albumin in agreement with that obtained above. This indicates that the low molecular weight estimates for this protein of Sørensen are to be ascribed to the high concentration of protein and salt in his experiments, and not to the decomposition of protein suggested by Svedberg.

The osmotic pressure of serum albumin was studied in 75 per cent glycerol solutions. The molecular weight calculated from these measurements was the same as in aqueous solution, within the limits of experimental error. Serum albumin is not denatured by glycerol solutions.

The minimal molecular weight of serum albumin calculated by the procedure of Cohn²⁰ from the recent determination of its tryptophane content by Folin and Marenzi²¹ leads to a value of 78,000 for the probable molecular weight of serum albumin, if it contains 2 tryptophane molecules.

¹⁹ Burk, N. F., and Greenberg, D. M., *J. Biol. Chem.*, **87**, 197 (1930).

²⁰ Cohn, E. J., Hendry, J. L., and Prentiss, A. M., *J. Biol. Chem.*, **63**, 721 (1925).

²¹ Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 89 (1929).

THE ISOELECTRIC POINT OF A STANDARD GELATIN
PREPARATION

BY DAVID I. HITCHCOCK

(From the Department of Physiology, Yale University, New Haven)

The material studied had been prepared according to the tentative specifications of the committee on standard gelatin of the Leather and Gelatin Division of the American Chemical Society,²² and freed from electrolytes according to Northrop and Kunitz.²³ The isoelectric point of this gelatin was determined by four methods, all involving pH determinations by the hydrogen electrode at 30°.

1. Solutions of the gelatin in distilled water, of concentrations from 1 to 12 per cent, were found to have pH values which approached the limit 4.86 ± 0.01 as the concentration of the gelatin was increased.

2. Measurements of pH in acetic acid-sodium acetate buffer solutions, with and without gelatin, showed no change in pH, due to the presence of gelatin, only at $\text{pH } 4.85 \pm 0.01$. This value was independent of the concentration of gelatin (1 to 4 per cent) and of salt (0.001 to 0.105 M).

3. 1 per cent solutions were made up with pH varied by the presence of small amounts of HCl or NaOH, or of 0.001 M Na acetate with varied amounts of acetic acid. On cooling, jellies were formed which exhibited maximum turbidity at $\text{pH } 4.85 \pm 0.05$.

4. Collodion particles were coated with gelatin by immersion in 0.01 or 0.1 per cent gelatin solutions made up in acetate buffers of ionic strength varied from 0.001 to 0.04. Microscopic measurements of the velocity of cataphoresis of such particles gave curves which located the pH of zero migration within 0.01 or 0.02 pH unit for each concentration of sodium acetate. The pH value of the cataphoretic isoelectric point was found to be 4.80 ± 0.01 , showing no definite trend with changes in salt concentration (0.04 to 0.001 M).

²² Davis, C. E., Sheppard, S. E., and Briefer, M., *Ind. and Eng. Chem., Anal. Ed.*, **1**, 56 (1929). Hudson, J. H., and Sheppard, S. E., *Ind. and Eng. Chem.*, **21**, 263 (1929).

²³ Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, **11**, 477 (1927-28).

No evidence was found for the assumption of certain previous workers that gelatin has two isoelectric points at widely separated pH values.

It is concluded that the isoelectric point of this gelatin is at pH 4.85, since the first three methods are believed to be more certain than the cataphoretic method with collodion particles.

EXPERIMENTS ON THE DIFFUSIBILITY OF PLASMA PROTEINS

By OLIVER HENRY GAEBLER

(From the Department of Laboratories, Henry Ford Hospital, Detroit)

In experiments with flat collodion membranes of high permeability it was found that the permeability of such membranes to protein is widely different in the two directions. Much less protein is transmitted if the side of the membrane which was uppermost, while the membrane was being formed on a glass plate, is placed toward the protein solution than if the reverse arrangement is made. This is especially true of membranes plunged directly into water after the collodion solution has evaporated to the desired extent. When the membranes are passed through a series of alcohol solutions of decreasing strength before water immersion, as suggested by Nelson and Morgan, the membranes become far more uniform, and transmit commercial egg albumin equally in both directions, but retain their asymmetry for plasma proteins.

Since an increased diffusibility of the plasma proteins, due to the effect of other substances either upon the proteins or upon the membranes, is postulated in several theories concerning albuminuria, dialyses with uniform membranes were carried out on plasma from subjects with and without albuminuria, and on plasma from dogs with and without nitrogen retention. Preliminary results do not show any increased diffusibility of the plasma proteins in the pathological conditions. Variations which do occur appear to be related to changes in the concentration of the individual proteins of the plasma.

THE ELECTRICAL FORCES IN SYSTEMS CONTAINING BIOLOGICAL COMPONENTS**I. THE SOLUBILITY OF ALIPHATIC AMINO ACIDS IN ALCOHOL-WATER MIXTURES CONTAINING NEUTRAL SALTS**

By EDWIN J. COHN, THOMAS L. McMEEKIN, JOHN T. EDSALL, AND JOHN H. WEARE

(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston)

Molecular interaction in concentrated solutions greatly influences the behavior of the individual components. The resultant forces constitute an element in the physiological environment. In biochemistry they render difficult the quantitative separation of chemical individuals from mixtures of nitrogenous substances and interfere with their crystallization.

It has been amply demonstrated by Bjerrum and others that amino acids are to be regarded as *Zwitter Ionen*; hence they are dipoles of very high electric moment due to the relatively large distance between their amino and carboxyl groups. The behavior of *Zwitter Ionen* is comparable in certain aspects, though not in all, to that of ions. Debye and Hückel have formulated a theory of strong electrolytes in terms of ionic strength, μ ; valence, Z ; ionic radii, b ; the mean distance between ionic centers at collision, a ; and the contribution, β , of each component, to the dielectric constant, D , of the solution. These theoretical advances suggested the study of systems containing numerous biological components, and the estimation of the electrical forces between them.

The studies reported upon amino acids and proteins yield constants in terms of which behavior may be quantitatively described in mixed solutions of electrolytes and non-electrolytes. Bjerrum, in 1923, demonstrated that glycine increased the solubility of other electrolytes. Conversely it has been shown that neutral salts increase the solubility of glycine. Both of these phenomena may be described in terms of an apparent valence type, which depends not only upon the number of charges, but the distance between them.

Neutral salts increase the ionic strength and diminish the dielectric constant of solutions. Certain amino acids and proteins

increase both the electrical forces and the dielectric constant. In systems containing such substances the rise in dielectric constant supplements interionic forces and increases solubility. Whereas neutral salts increase the ionic strength, the diminution in dielectric constant leads to salting out.

Solubility is also decreased by non-electrolytes which diminish the dielectric constant. Glycine exists predominantly as charged molecules in solutions containing less than 90 volumes per cent of alcohol. The logarithm of the solubility in alcohol-water mixtures diminishes proportionately with the relative lowering of the dielectric constant, $\frac{D_0}{D} - 1$, in the range in which it is not contributing largely to the dielectric constant. The proportionality constant yields the ratio of the apparent valence type to the electrical radius. This quantity has now been estimated for certain amino acids and proteins. For glycine the proportionality constant is 1.3, for alanine 1.0, and for cystine 5.5.

In systems of low dielectric constant interionic forces are amplified whereas in systems of high dielectric constant they are diminished. The solv nt action of neutral salts upon amino acids in alcohol-water mixtures has now been studied. Equations defining the influence of the ionic strength and of the dielectric constant in such systems have been developed by Debye, by H ckel, by Bjerrum, and by Scatchard. By their use estimates of the above constants have been derived from solubility measurements.

Leucine with its long branched hydrocarbon chain affects and is affected by interionic forces far less than glycine. Thus sodium chloride increases the solubility of glycine and decreases that of leucine. A fundamental difference in the behavior of the series of aliphatic amino acids of identical dissociation constants, and heats of neutralization, is thus apparent. Moreover the linear relation between $\log S$ and $\frac{D_0}{D}$ does not hold for leucine. Comparable differences between these amino acids are revealed also by the freezing point measurements of Frankel.

As a preliminary in apprehending the forces that obtain in the physiological environment, we have measured the solubility of

cystine in glycine solutions, which may be explained in terms of the same relations that have been deduced from the interaction of these amino acids, proteins, and other electrolytes. The interpretation of molecular forces between amino acids, proteins, and salts thus depends upon knowledge of their apparent valence, which depends in turn upon the number of charges and the distance between them, upon their electrical size, and contribution to the ionic strength and dielectric constants of solutions.

THE RATE OF AUTOXIDATION OF OXIDATION-REDUCTION SYSTEMS AND ITS RELATION TO THEIR FREE ENERGY

By E. S. GUZMAN BARRON

(From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago)

The catalytic effect of reversible dyes on cellular oxygen consumption has been shown to be conditioned by two factors: the reduction potential of the dye and the permeability of the cell surface. This relationship seemed in contradiction with the well known thermodynamic assumption that there is in general no distinct relationship between the potential and the velocity of reaction. This problem has been studied on homogeneous systems, namely the rate of oxidation of reversible systems by atmospheric oxygen, an electrometric method being used for that purpose. When the substance was in the oxidized state it was reduced with colloidal palladium and hydrogen. The amount of oxidation was determined by application of the well known Peter's equation. Gold and graphite electrodes were used for the measurement of the E.M.F. To avoid uncontrollable catalytic effects, only one kind of buffer was used in all experiments (citrate-NaOH or citrate-HCl) with the exception of hydroquinone, where phosphate buffers were employed. The measurement of the rate of oxidation was started when 2 per cent of the reduced substance had been oxidized and was followed until 50 per cent oxidation. Several dyes (phenolindophenol, O-cresol, 2,6-dichlorophenolindophenol, toluylene blue, 1-naphthol-2-sulfonate indophenol, cresyl blue, indigo tetrasulfonate) and some quinone derivatives (hydroquinone, benzidine) have been studied. If one takes into consideration the dyes only, the velocity of autoxidation is a simple

linear function of the available free energy of the system. This does not hold for the autoxidation of quinones where the rate of oxidation is considerably slower than expected.

When the rate of oxidation of single systems is studied, this relation is more clearly shown as the velocity of the reaction follows, between certain limits, the equation of monomolecular reactions.

INDUCED OXIDATIONS IN BLOOD. ACCELERATION OF RESPIRATION OF RED BLOOD CELLS BY CYANIDE

By W. B. WENDEL

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis)

Upon continuing the study of oxidation of lactate to pyruvate by (dog) red blood cells in the presence of methylene blue,²⁴ the anomalous observation is made that buffered cyanide (0.02 M) markedly increases the rate of oxidation. In the absence of methylene blue this concentration of cyanide is without effect. Higher concentrations of cyanide (0.1 M) in the absence of methylene blue cause oxidation of lactate to pyruvate by red blood cells. Cyanide (or pyridine) added to oxyhemoglobin solutions causes progressive loss of O₂ capacity, the rate increasing with increasing concentration of cyanide (or pyridine). The effect of low concentrations of cyanide is greatly accentuated by small amounts of hemin, which alone acts very slowly. This fact suggests that the acceleration of lactate oxidation by cyanide may be due to the formation of cyanhemochromogen (besides cyanmethemoglobin) which either acts within the cell directly as an oxidant of (activated) lactate, or catalyzes the oxidation by methemoglobin.²⁵ In any case, cyanide instead of blocking an oxidation by molecular O₂ (apparently through hemin iron) markedly accelerates it.

²⁴ Wendel, W. B., *Proc. Soc. Exp. Biol. and Med.*, **26**, 865 (1929); **27**, 624 (1930); **28**, 401 (1930). Wendel, W. B., and Shaffer, P. A., *J. Biol. Chem.*, **87**, p. xx (1930).

²⁵ Warburg, O., Kubowitz, F., and Christian W., *Biochem. Z.*, **221**, 494 (1930); **227**, 245 (1930).

**SOURCES OF ERROR IN EMPLOYING THE CULLEN CORRECTION
WITH THE COLORIMETRIC METHOD OF ESTIMATING
PLASMA pH**

BY EDWARD MUNTWYLER AND VICTOR C. MYERS

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland)

The colorimetric method for the estimation of the hydrogen ion concentration of the blood, employing the Cullen correction, has received some criticism when applied to pathological bloods. Stadie, Austin, and Robinson²⁶ have pointed out that the error in the colorimetric method is due to changes in the Cullen factor, which corrects the pH from 20 to 38°, in that this correction varies with different plasma depending upon the pH range, CO₂ content, and protein concentration.

Since the ease and rapidity with which the colorimetric method may be performed and the requirement of only small amounts of material makes it preferable in clinical work, or experimental work where only small amounts of material are available, it seemed desirable to study the magnitude of the error in the Cullen correction with changes in protein concentration and pH. This was done by comparing the colorimetric and electrometric methods of determining pH on CO₂-equilibrated samples of serum or plasma and serum diluted so as to give varying protein and constant salt concentrations. In agreement with the above workers²⁶ it has been shown that the Cullen correction shows a definite variation with changes in pH and protein concentration. With "true plasma" samples, obtained from equilibrating whole blood with different CO₂ tensions, the C correction showed a maximum difference of 0.14 pH in two determinations on the same sample equilibrated with CO₂ to give pH, 38° values of 7.38 and 7.14 respectively. Since as has been pointed out by Stadie, Austin, and Robinson²⁶ the C correction is lowered with an increase in total CO₂ at a given protein concentration and pH, which is in agreement with our observations, this factor no doubt augments the difference in the C corrections observed. Pooled human serum and pooled dog plasma were diluted with salt solutions, thus

²⁶ Stadie, W. C., Austin, J. H., and Robinson, H. W., *J. Biol. Chem.*, **66**, 901 (1925).

varying the protein concentration, but keeping the total salt concentration constant. Equilibrating such samples it was observed that the C and T (pH_c 20° — pH_c 38°) corrections varied with the protein concentration and also the pH. An increase in the pH caused an increase in the C correction, while a decrease in the protein caused a decrease in the C correction. The H (Hastings and Sendroy²⁷) correction for the pooled human serum remained quite constant with the variations in pH and protein concentration. The H correction was not so constant, however, when the pooled dog plasma was employed, and showed a maximum discrepancy of -0.10 pH.

It would appear that the reason why some observers, for example, Myers and Muntwyler,²⁸ have reported fairly constant findings for the C correction on hospital bloods is due to the fact that the factors affecting the C correction tend to oppose each other; *i.e.*, a high pH accompanies a high total CO_2 and *vice versa*.

DETERMINATION OF THE pH OF WHOLE BLOOD AS WELL AS OF SERUM WITH QUINHYDRONE

By MARTIN E. HANKE

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New York)

The direction of the drift in potential observed in the customary pH measurement of serum with quinhydrone has been shown to depend upon the concentration of quinhydrone; high concentrations of quinhydrone producing an acid drift, and very low concentrations an alkaline drift. By choosing suitably the quinhydrone concentration it is possible to eliminate the drift entirely with whole blood as well as with serum. Moreover the absolute value of the potential can be regulated within limits (about 5 millivolts for serum and 20 millivolts for blood) by controlling the salt and protein concentrations, and correct pH values can be obtained by balancing the alkaline protein error with an acid salt error. The simultaneous elimination of the drift and control of potential, involving the simultaneous control of all three concentrations (salt, protein, and quinhydrone), is effected by diluting

²⁷ Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, **61**, 695 (1924).

²⁸ Myers, V. C., and Muntwyler, E., *J. Biol. Chem.*, **78**, 243 (1928).

the serum or blood with carefully measured volumes of quinhydrone in aqueous sodium chloride solutions.

For normal dog or beef serum the best dilution for eliminating the drift and making the calculated pH match the hydrogen electrode value is 1 volume of serum to about 1 volume of saturated quinhydrone in 0.9 per cent aqueous sodium chloride. In order to match the hydrogen electrode pH with whole blood, it is necessary to dilute 1 volume of blood with about 30 volumes of one-half saturated quinhydrone in 5 per cent sodium chloride, and the difficulty of measuring these high dilutions accurately in the electrode vessel has so far given variable results. Constant and easily duplicable values can, however, be obtained with whole blood by diluting 1 volume of blood with about 3.5 volumes of saturated quinhydrone in 0.9 per cent sodium chloride. The pH thus calculated is uniformly about 0.2 more alkaline than the hydrogen electrode value. The exact choice of dilution for eliminating the drift varies slightly in different bloods and sera, depending upon their protein concentration, and must be determined by one or two preliminary trials.

Determinations were made on eight different samples of normal beef blood and one of normal dog blood in which the hydrogen electrode pH on the serum was compared with the quinhydrone pH on the serum and the quinhydrone pH on whole blood. A saturated solution of quinhydrone in 0.9 per cent NaCl was used, and the serum and blood were diluted with this so that a constant potential was obtained. To the quinhydrone values on whole blood a correction of -0.20 pH was applied to compensate for the alkaline protein error. With serum the maximum deviation of the quinhydrone pH from the hydrogen electrode value was 0.02 pH, while with whole blood it was 0.05 pH. These deviations are due to variations in the protein content of the sera and bloods. With bloods or sera of the same protein content, differences in pH can be determined just as accurately with this quinhydrone dilution method on whole blood or serum, as they can with the hydrogen electrode, to within 0.005 pH. A complete determination with this quinhydrone dilution method can be made in 5 minutes.

For this work quinhydrone electrode vessels of the Cullen and Biilmann type (a glass tube with a thin 2 cm. platinum wire

sealed in one end, moving as a plunger inside a larger tube holding the liquid) were used, which were graduated to 0.01 cc. so that dilutions could be read accurately. The hydrogen electrode pH values were determined in a Clark electrode assembly. Both systems were independently standardized with the same 0.1 N HCl daily.

THE INFLUENCE OF ELECTROLYTES ON THE GELATION OF METHYLAMINE URATE

By E. GORDON YOUNG AND FORREST F. MUSGRAVE

(From the Department of Biochemistry, Dalhousie University, Halifax, Canada)

The effect of various electrolytes in promoting gelation of aqueous supersaturated solutions of methylamine urate has been studied. The cation has been shown to be the active agent. The minimal concentration of the cation which is effective depends upon its valence, as in the case of the coagulation of typical suspensoids. Salts of sodium, potassium, ammonium, and rubidium have been found to induce gelation. Salts of lithium and cesium were negative in their effect.

METABOLIC EFFECTS OF PROLONGED ADMINISTRATION OF EPINEPHRINE

By ALFRED E. KOEHLER, FRITZ BISCHOFF, AND ELSIE HILL

(From the Chemical Laboratory of the Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara)

The transient nature of the effect of epinephrine continuously administered on blood sugar and blood lactic acid has been demonstrated. All experiments to our knowledge in regard to the effect of epinephrine when continuously or repeatedly administered on the basal metabolism have not extended beyond half an hour. The respiratory quotient observations have also extended only over short periods when the markedly increased respiratory volume caused excessive output of CO₂ and consequently high respiratory quotients.

The experiments to be reported concern the effects of single and repeated small doses of epinephrine over extended periods of

time on the basal metabolism, respiratory quotients, and nitrogen metabolism, together with simultaneous observations on the blood lactic acid, blood sugar, and blood fat.

THE INFLUENCE OF EPINEPHRINE AND INSULIN ON THE HEXOSE-PHOSPHATE CONTENT OF MUSCLE

BY CARL F. CORI AND GERTY T. CORI

(From the State Institute for the Study of Malignant Disease, Buffalo)

It is known that injections of insulin and also of epinephrine lead to a diminution in urinary phosphate excretion. The explanation usually offered is that phosphate is held in some organic combination in the tissues and in the case of insulin it has been suggested that it might be hexosephosphate. Conclusive evidence for or against this assumption could not be obtained because of the lack of a satisfactory method for the determination of this compound. A method recently elaborated by the authors permits the separation of hexosemonophosphate from other phosphorus compounds present in an acid extract of muscle and its simultaneous determination as hexose and as P. The P content, when calculated from the hexose content, generally checks very closely with the P value as actually determined. Added hexosephosphate is recovered satisfactorily by this method. After subcutaneous injection of epinephrine in rats (0.02 mg. per 100 gm.) the hexosephosphate content is definitely increased 15 minutes after injection, reaches twice its normal value after 30 minutes, remains at this level until 1 hour, and then slowly returns to the original level in the next 2 to 3 hours. An increase of nearly equal magnitude is obtained when the animals are under amytal anesthesia, showing that muscular activity is not involved. Since previous work has shown that epinephrine injections cause a decrease in muscle glycogen and increase in blood lactic acid of rats and other species, hexosemonophosphate appears here as an intermediary of this transformation. Hydrolysis curves in N HCl gave no evidence for the accumulation of a hexosediphosphate. The increase in hexosephosphate throughout the muscles of the body is large enough to account for the decrease in phosphate excretion after epinephrine injections. Insulin injections in fasted rats produce a marked increase in hexosemonophosphate

in about 1 hour. Since this effect does not occur (a) when hypoglycemia is prevented by simultaneous administration of glucose and (b) when insulin is injected into adrenalectomized rats, it may be concluded that the increase in hexosephosphate is not due to insulin but to a secondary output of epinephrine elicited by the hypoglycemia. The latter need not be severe, since in some cases evidence for adrenal discharge was obtained at an arterial plasma sugar level of 65 mg. per cent. Insulin injections alone or in combination with glucose produced no measurable change in the pyrophosphate fraction of muscle. Glucose feeding was not followed by an increase in the hexosephosphate content of muscle. The decrease in the excretion of urinary phosphates after a carbohydrate meal thus remains unexplained. After 5 seconds of stimulation of the nerve with a tetanizing current the hexosemonophosphate content of the rat gastrocnemius was found to have increased from an average resting value of 52 mg. per cent (as hexose) to 95 mg. per cent. After 10 seconds of stimulation the value rose to 117 mg. per cent and did not increase any further even if the stimulation was continued for 1 minute. The hexosephosphate content returned to the original level about 10 minutes after terminating the stimulation.

SOME PECULIARITIES OF THE INSULIN REACTION IN HUMANS

By LILLIAN A. CHASE

(From Regina, Saskatchewan, Canada)

For 6 months a patient had night overdoses of insulin with resulting violent night reactions. When all insulin was withdrawn night hypoglycemic reactions continued. If the patient's activities were less than usual, vomiting supervened and she became a typical severe diabetic with high blood sugar requiring large doses of insulin. It is suggested that due to habitual overstimulation by insulin there is either a lessened storage of liver glycogen and of tissue glycogen, or there is a hypersecretion of insulin. The former supposition seems more probable. In another 6 months after sugar had been given every night, the reactions ceased to occur.

THE "HEAT PRECIPITATE" OF CRYSTALLINE INSULIN

BY VINCENT DU VIGNEAUD

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana)

The only reaction of insulin which so far seems to offer the possibility of being characteristic or peculiar to insulin is the formation of the "heat precipitate." It has previously been reported²⁹ that crystalline insulin dissolved in 0.1 N HCl yields a flocculent precipitate when heated in a boiling water bath. The insolubility of this precipitate in dilute acid and the recovery of its solubility in this medium after solution in alkali has already been noted. In the present investigation the formation of the precipitate, its solubility and properties, and particularly its relationship to the activity of insulin have been more closely studied.

In the present work it has been found that the reversal of the solubility of the heat precipitate can also be brought about by 20 per cent HCl. The heat precipitate dissolves in the concentrated acid but in so doing it regains its solubility in dilute acid. The cycle can be repeated, the insulin precipitated from the dilute acid by heat, etc.

An attempt was made to crystallize the heat-precipitated material, the solubility of which in dilute acid had been regained by the dilute alkali treatment. Our results confirmed those of Jensen³⁰ in that no crystals were obtained. Either split-products are formed which prevent the remaining intact insulin from crystallizing or else the material resulting from the treatment has been so changed that it no longer is crystallizable under the same conditions although it is still active.

In all the cases that we have so far studied in which insulin has been inactivated by reduction, the resulting inactive products have lost the ability to yield the heat precipitate under the conditions mentioned above. We have found for instance that insulin is inactivated by glutathione and by cysteine. The resulting inactive products are not precipitable by heat like the original

²⁹ du Vigneaud, V., Geiling, E. M. K., and Eddy, C. A., *J. Pharmacol. and Exp. Therap.*, **33**, 497 (1928).

³⁰ Jensen, H., and De Lawder, A., *Z. physiol. Chem.*, **190**, 262 (1930).

crystalline insulin. The same is also true for the products inactivated by sodium cyanide and hydrogen sulfide.

The above results are rather suggestive of the possibility that the groups involved in the physiological action might be those necessary as well for the formation of the heat precipitate. Further investigations are therefore under way to see if loss in physiological activity is always associated with a loss in ability to yield the heat precipitate.

STUDIES ON THE PLASMA CALCIUM-RAISING PRINCIPLE OF BOVINE PARATHYROID GLANDS

II. ATTEMPTS AT PURIFICATION

BY WILBUR R. TWEEDY AND J. J. SMULLEN

(From the Department of Physiological Chemistry, Loyola University School of Medicine, Chicago)

In a previous publication,³¹ a method was described for the preparation of a plasma calcium-increasing principle of high, but variable, potency.

Subsequent work has further demonstrated that in common with Abel and Geiling's experience³² with commercial insulin, a 90 per cent aqueous solution of phenol is an excellent solvent for parathyroid proteins.

Furthermore, it has been found possible to fractionate the more inert proteins from a 20 per cent solution in 90 per cent phenol by the addition of alcohol to a concentration of 75 per cent. Upon the addition of an equal volume of ether, a highly active fraction is precipitated, representing in weight 50 per cent, or less, of the original material depending upon the activity of the starting material.

The active fraction gives the common protein color reactions with the exception of the ninhydrin and reduced sulfur tests. It also gives a positive phosphorus test and a positive Molisch test.

A uniformly highly active dry preparation is thus obtained which has been found to retain its original potency after several months storage over anhydrous calcium chloride. Attempts at further purification are in progress.

³¹ Tweedy, W. R., *J. Biol. Chem.*, **88**, 649 (1930).

³² Abel, J. J., and Geiling, E. M. K., *J. Pharmacol. and Exp. Therap.*, **25**, 423 (1925).

A CHEMICAL STUDY OF THE ACTIVE CONSTITUENTS OF THE SUPRARENAL GLAND

BY EDWARD C. KENDALL

(From the Division of Chemistry, The Mayo Foundation, Rochester, Minnesota)

Epinephrine, hexuronic acid, and lactic acid may be separated from the suprarenal by the following method.

The frozen glands are ground and added directly to acetone which has been cooled to 0°. The cc. of acetone should be equal to the weight of the glands in gm. The suspension is stirred occasionally for 3 hours and the acetone solution is then pressed from the residue as completely as possible. The acetone solution is concentrated to a small volume, is made acid with sulfuric acid to a pH of about 2, and is extracted with ether in a continuous extractor. About 0.2 per cent of the weight of the raw material is lactic acid. The solution is then treated with phosphotungstic acid, the excess of which is removed with zinc dust and barium acetate. Barium is removed and the solution is concentrated to a small volume. The epinephrine is precipitated with ammonia in an atmosphere free from oxygen. This step is carried out without loss of hexuronic acid which is contained in the filtrate. The ammoniacal solution is added to a solution of oxygen-free lead acetate. The lead salt is decomposed with oxalic acid. The solution is concentrated and treated with methyl alcohol and acetone. This removes inorganic salts and traces of epinephrine. The solution is concentrated to small volume and the residue thoroughly dried in a high vacuum at 40°. A small amount of methyl alcohol is added. The hexuronic acid crystallizes after several days.

METABOLISM STUDIES ON THE ADRENAL CORTICAL HORMONE

BY GEORGE A. HARROP, JR., W. W. SWINGLE, AND J. J. PFIFFNER

(From the Johns Hopkins Hospital, Baltimore, and the Laboratory of Biology, Princeton University, Princeton)

The studies as described by Swingle and Pffner we have to report were all done with the standard cortical extract, 1 cc. of which represents the material from about 30 gm. of fresh gland.

A number of injections have been made in men in doses ranging

from 15 to 30 cc. per dose without any untoward effects. Many complain, after repeated doses, of an unpleasant musty taste and smell immediately after injection. There are no other clinical symptoms: no pulse changes, blood pressure effects, nor febrile reaction. The effect on the basal metabolism is not constant or striking either in those whose basal rate is within normal limits or whose rate is subnormal. A transitory rise sometimes occurs shortly after the injection of 20 to 30 cc., which is however undoubtedly an adrenalin effect as it can be approximately duplicated by the injection of a similar volume of a solution containing 1 part of adrenalin in 1.5 to 2 million parts of solution. Such injections produce no definite changes in non-protein nitrogen, blood sugar, inorganic phosphates, cholesterol, or plasma proteins, nor is there any change in the oxygen capacity of the blood.

Massive single doses of the cortical extract ranging from 25 to 100 cc. have been injected intravenously into normal dogs without the slightest effect during the following 24 hours on non-protein nitrogen, urea nitrogen, plasma proteins, blood sugar, inorganic phosphates, cholesterol, chlorides, erythrocyte count, blood oxygen capacity, or hematocrit. Following the injection of 100 cc. of the extract intravenously into a dog of 15 kilos there was transitory dyspnea and bradycardia (50 to 60) for about 40 minutes and the dog seemed somewhat uneasy and restless. Thereafter he appeared perfectly normal.

A considerable series of bilaterally adrenalectomized dogs has been studied. The earliest constant symptom of insufficiency is refusal of food. Coincident with this and usually preceding it there is a sharp rise in blood non-protein nitrogen and a proportionately greater rise in urea nitrogen. These phenomena antedate a change in the respiratory metabolism. A series of animals maintained under standard conditions were injected with daily subcutaneous doses of the extract, gradually diminishing the per kilo dosage in steps at intervals of 5 days. It is found that they can be kept in apparently normal condition, without change in blood non-protein nitrogen, on a minimal dose of $\frac{1}{3}$ cc. per kilo. One dog showed no evidence of insufficiency for 5 days on $\frac{1}{3}$ cc. per kilo, but in most instances prompt changes occur. We therefore regard $\frac{1}{3}$ cc. per kilo per day as the minimal maintenance dose for dogs.

Dogs have been repeatedly brought into insufficiency either through the abrupt or the gradual withdrawal of the extract. In all cases a characteristic set of changes occurs in the freshly drawn arterial blood taken from the animal which has been lying at rest on the table for at least 2 hours. Proportional to the severity of the insufficiency there is a rise in non-protein nitrogen with an increase in the urea nitrogen : non-protein nitrogen ratio. Later there is a rise in serum phosphorus and a slight rise in serum calcium, with a moderate lowering of the blood sugar. The blood lactic acid tends to increase and the potassium increases somewhat.

The increase in blood concentration with changes in plasma proteins, acidosis, etc. is a terminal phenomenon, not to be confused with the earlier stages. When the condition of insufficiency is terminated by large doses of cortical extract there is a gradual disappearance of all of the adequate blood chemical abnormalities, within 72 hours. The condition is more difficult to influence and the abnormalities clear up more slowly after repeated periods of insufficiency in the animal, and in the presence of infection. The latter is particularly difficult to overcome in spite of the exhibition of large amounts of the cortical extract.

FURTHER STUDIES IN THE RELATIONSHIP BETWEEN THYROXINE AND BILE SALTS

By SHIRO TASHIRO AND L. H. SCHMIDT

(From the Department of Biochemistry, University of Cincinnati, Cincinnati)

The previous finding that hyperthyroidism will aggravate the toxic action of the bile salts in producing gastric ulcer was based on the assumption that thyroxine has the power to diminish the amount of blood phospholipids, since these are the only known compounds that have the power to antagonize the ulcer-producing action of the bile salts. This report is to show that not only is this decrease in phospholipids produced by hyperthyroidism, but also that it is produced by several other agencies, such as diphtheria toxin and adrenalin, which are known to produce gastric ulcer. Since bile salts themselves have the power to diminish this antagonist, the question whether the production of gastric ulcer is due to the aggravated activity of the bile salt, due to the

decrease in antagonist, or due to the decrease in phospholipids *per se* cannot be determined until it is known whether or not normal blood contains bile salt.

ON THE ORIGIN OF CREATINE. III

BY ERWIN BRAND AND MEYER M. HARRIS

(From the Departments of Chemistry and of Internal Medicine, New York State Psychiatric Institute and Hospital, Columbia-Presbyterian Medical Center, New York)

In some previous publications³³ it was shown that glycine may give rise to creatine. It is suggested that this amino acid may furnish either 1 or 2 of the nitrogen atoms of the guanidine group of creatine as follows: (1) Glycine reacting with a urea precursor or some form of urea (urea *in statu nascendi*, isourea, cycloammonium amide urea) would yield guanidoacetic acid, which is then methylated to creatine (40 to 50 per cent methylation of guanidoacetic acid has been obtained in cases of muscular dystrophy). However, there is no evidence of the formation of guanidoacetic acid in intermediary metabolism. (2) 2 glycine + cyanic acid = creatine + CO₂ + H₂O.

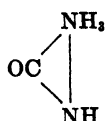
According to this equation the methyl group of creatine is derived from decarboxylation of 1 molecule of glycine. The location of the methyl group thus obtained, however, would not be in agreement with that accepted for the naturally occurring creatine.

Investigations are at present in progress regarding the constitution of creatine. The migration of a methyl group to form the more stable naturally occurring compound is one of the possibilities in regard to which evidence is being sought.

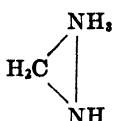
In regard to the constitution of urea and its derivatives, such as thiourea, hydantoic acid, guanidine, creatine, arginine, etc., it should be noted that they may be looked upon as derivatives of a cyclic form of ammonium amide³⁴ (NH₄·NH₂).

³³ Brand, E., Harris, M. M., Sandberg, M., and Ringer, A. I., *Am. J. Physiol.*, **90**, 296 (1929). Brand, E., Harris, M. M., Sandberg, M., and Lasker, M. M., *J. Biol. Chem.*, **87**, p. ix (1930).

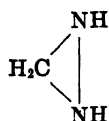
³⁴ Werner, E. A., in *Chemistry of urea*, London, 138 (1923), is inclined to disregard this formula.



Urea



Ammonamminemethylene

Hydrazomethylene³⁵

Our studies on the ester hydrochlorides of guanidoacetic acid, of creatine, and of isocreatine (*n*-methylcarboxymethylguanidine) indicate that they are real esters and not cyclic anhydrides containing alcohol in some unknown form of combination.³⁶

The ease with which such derivatives of the open compounds are converted directly into the anhydrides renders these esters of considerable physiological interest. Such compounds occurring in biological material would be accounted for as creatinine with the methods now available, and we can no longer be certain in our creatinine determinations that that is the compound which is actually present (*e.g.*, in urine).

THE EXTRACTIVES OF DOG MUSCLE

BY WILLIAM A. WOLFF AND D. WRIGHT WILSON

(From the Department of Physiological Chemistry, School of Medicine,
University of Pennsylvania, Philadelphia)

Anserine, a dipeptide of β -alanine and methylhistidine, has been isolated by us from dog muscle. This is the first time the compound has been found in mammalian muscle. It was first isolated in 1929, by Ackermann, Timpe, and Poller,³⁷ from the muscles of birds and reptiles. The presence of anserine in dog muscle which is low in carnosine suggests that it may replace carnosine in this as well as in muscles of other animals.

The compound obtained from dog muscle was identified as

³⁵ Meyer, V., and Jacobson, P. H., *Lehrbuch der organischen Chemie*, Berlin, 2, pt. 3, 39 (1920).

³⁶ Hunter, A., in *Creatine and creatinine*, London and New York, 38 (1928), classifies these esters as creatinine derivatives. K. Thomas, in whose laboratory the work of Kapfhammer (Kapfhammer, J., *Biochem. Z.*, 156, 192 (1925)) was carried out, agrees that these esters are probably creatine derivatives (personal communication).

³⁷ Ackermann, D., Timpe, O., and Poller, K., *Z. physiol. Chem.*, 183, 1 (1929).

anserine as follows: The material was precipitated from the lysine fraction of the muscle extract by HgSO_4 and H_2SO_4 in the presence of alcohol. After several precipitations, a copper salt was formed which crystallized in needles and was lilac in color when dried in air. The nitrate and free base were also prepared. The three compounds had melting points similar to those reported by the German investigators. Data for total nitrogen, amino nitrogen, and methylimino nitrogen agreed satisfactorily with the calculated values.

THE RELIEF OF ANEMIA, DUE TO MILK DIET, BY FEEDING AMINO ACIDS AND RELATED COMPOUNDS

BY DAVID L. DRABKIN AND H. K. MILLER

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

The experimental work upon the relief of anemia, due to milk diet, in rats by feeding organic substances³⁸ has been continued. As in the previous work, at the height of the anemia the milk was supplemented by a small amount of iron salt (in itself insufficient to check the anemia) and by various amino acids or related compounds. The quantities of supplements fed are expressed as 1, 2, or 4 equivalents on the basis of molecular weight to 70 mg. (a very effective level) of glutamic acid.

Under the experimental conditions, both succinic acid (2 equivalents) and succinimide (4 equivalents) have been found somewhat effective in the regeneration of hemoglobin. Leucine (1 equivalent), cystine (1 equivalent), glycine (4 equivalents), α -aminovaleric acid (2 equivalents), and glutaric acid (2 equivalents) have been found ineffective in the relief of milk anemia. As before, histological studies of the organs of the rats have been made. These studies have shown glutaric acid to be toxic for the rat.

Experiments upon the prevention of milk anemia indicated (a) that rats became anemic upon boiled milk as readily as upon raw milk, (b) that prevention of anemia was accomplished by the administration of an iron salt at a level which was ineffective in curing the anemia once produced, (c) that rats fed sodium

³⁸ Drabkin, D. L., and Miller, H. K., *J. Biol. Chem.*, **90**, 531 (1931).

glutamate were definitely more resistant to anemia, even in the absence of iron administration.

The quantity of glutamic acid (fed as sodium glutamate) and iron needed for the relief of milk anemia has been studied. With 0.2 mg. of iron, the feeding of 35 mg. of glutamic acid exerted a definite stimulus for hemoglobin regeneration. At this level of iron feeding, stimulation was also somewhat evident with 17.5 mg. of glutamic acid, while 8.8 mg. of the amino acid were ineffective. With 70 mg. of glutamic acid, there was a definite increase in hemoglobin with only 0.1 mg. of iron and there was a slight, temporary stimulation with as little as 0.05 mg. of iron supplement. Glutamic acid alone was ineffective.

A preliminary experiment upon several dogs has indicated that glutamic acid is also an effective stimulant for hemoglobin production in anemia following hemorrhage.

**THE ACTION OF IRON AND IRON SUPPLEMENTED WITH OTHER
ELEMENTS UPON THE RETICULOCYTE AND RED BLOOD
CELL RESPONSE IN THE NUTRITIONAL ANEMIA
OF THE RAT**

BY HOWARD H. BEARD AND VICTOR C. MYERS

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland)

The reticulocyte and red blood cell response has been studied daily in nutritional anemia of the rat under the influence of inorganic elements.

With 0.25 mg. of Fe alone the reticulocyte peak was reached in 4 days, with a second peak at 7 days, and a return to normal in 3 or 4 more days. Red blood cell recovery was slow and gradual. This indicated that Fe alone stimulates the erythropoietic organs to produce new reticulocytes.

When other elements were added to Fe there was no greater stimulation of the production of reticulocytes but the change of the reticulocytes into mature red blood cells was increased more than with Fe alone as shown by the quick drop in reticulocytes and quick increase in red blood cells. This suggests that the action of these elements with Fe brings about a quicker production of mature red blood cells from reticulocytes.

DIETARY FACTORS AFFECTING THE APPETITE AND GROWTH OF RATS

BY CLAIKE E. GRAHAM AND WENDELL H. GRIFFITH

(From the Department of Biochemistry, St. Louis University School of Medicine, St. Louis)

The rate of growth and the utilization of food in growing rats have been determined in order to throw additional light upon the relation of the vitamin B complex to growth. The Evans and Burr diet (purified casein, 25; sucrose, 75; salt mixture, 4) was used and was supplemented daily with 9 drops of cod liver oil. The vitamin B complex was furnished by additions of one or more of the following: liver, autoclaved liver, yeast, and an extract of rice polishings. Gain in weight during 60 day experimental periods was comparable to that of rats on the stock ration if the experimental diet was supplemented daily with 0.5 gm. of liver and 0.5 gm. of yeast or with the extract of rice polishings and 1.0 gm. of autoclaved liver. Rats fed the experimental diet supplemented with autoclaved liver (vitamin B₂) alone showed a marked failure of growth and decrease in food consumption. The effects were similar, although less marked, if the diet was supplemented with only the extract of rice polishings (vitamin B₁). An inadequate supply of either one or of both vitamins B₁ and B₂ resulted in decreased food consumption and decreased efficiency of utilization of the ingested food. The appetite of the rats appeared to be more easily affected than the utilization of food. In these experiments both vitamins B₁ and B₂ appeared to serve as appetite stimulants but only in the presence of the other.

A NEW TECHNIQUE FOR DETERMINING THE DIETARY FACTORS THAT INFLUENCE THE SECRETION OF MILK IN LABORATORY ANIMALS

BY M. S. KOZLOWSKA AND C. M. MCCAY

(From the Laboratory of Animal Nutrition, Cornell University, Ithaca)

The following three methods have been compared in order to determine their relative values in measuring the quantity and quality of the milk secreted by the rat under various dietary conditions: (1) replacing the litter of a suckling mother by a new and younger litter at regular intervals and thus prolonging

the lactation period; (2) forcing a mother rat to continue nursing her litter after the normal weaning time by a mechanical feeding arrangement whereby the mother can consume all the food she desires but the young can get no food other than the milk of their mother; (3) alternately feeding the nursing mother separately from the young and then permitting periods of nursing when neither young nor mother can obtain external food, thus forcing the young to exist upon their mother's milk alone during their entire lives.

The third technique has proved the most satisfactory. Under such experimental conditions litters that contain five or six young can attain a mean weight of 40 to 50 gm. and can be kept alive for 8 to 10 weeks, upon synthetic rations.

The effect of varying the protein level has been determined with a series of synthetic rations containing varying amounts of casein ranging from 10 to 40 per cent of the dry ration. Anemia of the young nursing rats was prevented by separate feeding of small amounts of iron, copper, and iodine. The higher protein levels showed greater secretion of milk and consumption of food by the nursing mother when the mother rat was allowed all the food she wished. When the amount of food consumed by the mothers upon the high protein diets was limited to that eaten by those upon the lower level, the increased milk secretion upon the high protein diet was still found, but was lower than under conditions of unrestricted food consumption.

FURTHER OBSERVATIONS ON EGG WHITE INJURY IN THE RAT

By HELEN T. PARSONS

*(From the Department of Home Economics, University of Wisconsin,
Madison)*

Dried egg white, when constituting 66 per cent of a well supplemented ration, has been shown to produce pellagra-like manifestations in rats.³⁹

Beef liver, highly effective as a cure when replacing 20 per cent of yeast in the ration, is one-half to three-fourths as potent after autoclaving at pH 8 for 5 hours at 15 pounds pressure, and becomes entirely ineffective on ashing. 30 per cent of yeast or of dried

³⁹ Parsons, H. T., *J. Biol. Chem.*, **90**, 351 (1931).

egg yolk is not curative. No improvement follows additions of copper or of iron, singly or together. The pellagrous manifestations almost entirely disappear, however, and rapid growth is resumed if the egg white of the ration fed is heated, moist, for 3 hours at 80°. The improvement is not due merely to coagulation, since egg white brought rapidly to the boiling point or heated for 20 minutes at 80° is not curative, although a hard clot is produced. Mild digestion with pepsin or with HCl alone at pH 2.4 also renders the egg white curative. Raw fresh egg white is not curative when it replaces 66 per cent of dried egg white, but on the contrary pellagrous manifestations are produced on it. Fresh egg white even barely coagulated quickly at 65–68°, however, is mildly curative.

STIMULATION OF ENDOGENOUS PROTEIN METABOLISM BY AMINO ACIDS

By JAMES MURRAY LUCK AND MAURICE R. AMSDEN

(From the Biochemical Laboratory, Stanford University, California)

In 1929, one of us reported to The Thirteenth International Physiological Congress that the subcutaneous administration of amino acids to rats is followed by a delayed formation of urea. Although amino nitrogen determinations on the whole animal indicated that *l*-aspartic acid, *d*-glutamic acid, *dl*-alanine, and glycine were fully catabolized within 10 hours or less, urea formation was ordinarily proceeding at a high level and was incomplete at the end of the 10 hour period (alanine excepted). It was also observed that during the first 4 or 5 hours urea formation lagged far behind the rate of amino acid disappearance but in some cases ultimately exceeded in quantity the amount calculated to have arisen from the injected amino acids. The behavior of alanine was again exceptional.

During the past year we have endeavored to determine whether the initial lag in urea formation is due to a depression exerted by the injected amino acids upon the endogenous rate of protein catabolism. We have investigated the problem by administering amino acids to dogs subcutaneously and analyzing the urine, collected in 2 hour periods, for amino acid nitrogen, urea, and total sulfate. We were interested to observe that even in a total

postinjection period of $7\frac{1}{2}$ hours the extra urea nitrogen excreted after the administration of glycine exceeded that which could have arisen from the injected amino acid (seven experiments). With aspartic acid (three experiments) the excess formation of urea was very great. After glutamic acid (five experiments) it was indicated that urea formation would exceed the quantity calculated to have arisen from the injected amino acid. After alanine (six experiments) the behavior was exceptional in that urea formation, even after $7\frac{1}{2}$ hours, was still far below the amount calculated for the catabolism of both isomers.

In all cases, the injection of amino acids was followed by a 30 to 90 per cent increase in the excretion of total sulfate.

We conclude, therefore, that parenterally administered amino acids stimulate endogenous protein catabolism. The initial lag in urea formation observed in the experiments on rats is apparently due to the formation of nitrogenous intermediates in which the nitrogen is present neither as urea nor as primary amino nitrogen, or to some other unrecognized factor.

FEEDING EXPERIMENTS WITH MIXTURES OF HIGHLY PURIFIED AMINO ACIDS. A PRELIMINARY REPORT

By WILLIAM C. ROSE, RUTH H. ELLIS, WALLACE WINDUS, AND FLORENCE L. CATHERWOOD

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana)

Feeding experiments have been conducted with diets in which the protein (other than that present in 0.2 gm. of yeast employed daily as a source of the vitamin B factors) has been replaced by a mixture of highly purified amino acids. When all of the well recognized protein components except hydroxyglutamic acid are incorporated in the food, rats receiving such diets rapidly lose weight. Even the addition of a protein fraction carrying the dicarboxylic amino acids fails to improve the quality of the food. The results point to the fact that growth-promoting proteins contain at least one component other than the twenty known amino acids.

When 5 per cent of casein, gliadin, or gelatin is substituted for an equivalent quantity of the amino acid mixture, the animals lose weight for approximately 4 days and then slowly gain. Casein

is more effective than either gliadin or gelatin in stimulating growth. This growth stimulation provides additional evidence for the existence of an unknown essential.

Attempts have been made to concentrate the effective substance. As a result, a fairly active material contaminated by large amounts of inactive amino acids has been separated from casein. This is being subjected to further fractionation.

PRELIMINARY STUDIES ON AMINO ACID TOXICITY AND AMINO ACID BALANCE

BY M. X. SULLIVAN, W. C. HESS, AND W. H. SEBRELL

(From the National Institute of Health, United States Public Health Service, Washington)

A diet containing casein and giving good growth of young albino rats was reduced in protein (casein) until subnormal growth occurred. To this low protein diet various amino acids were added at different levels. Cystine, at a 0.5 per cent level, gave excellent growth and the rats were in fine appearance and activity. At a 5 per cent level retardation of growth occurred with evidence of toxicity. At higher cystine levels as reported by Newburgh, serious injury to the liver and kidney was observed. Tyrosine replacing cystine gave good growth at a 2.5 per cent level but with some indication of eye trouble. At 5 per cent it was decidedly toxic and killed the rats in 1 week with swelling of the extremities, paralytic symptoms, and serious inflammation of the eyes. 5 per cent cystine fed conjointly with 5 per cent tyrosine delayed the pathological symptoms and in general greatly lengthened the span of life. A histological study of some of the organs is being made by Dr. R. D. Lillie.

THE NITROGEN AND AMINO ACID CONTENT OF VARIOUS TUBERCULINS

BY FLORENCE B. SEIBERT AND BETTY MUNDAY

(From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, the University of Chicago, Chicago)

An attempt was made to determine whether the potencies of various tuberculo-protein preparations purified in different ways, which we now have in powder form, paralleled their nitrogen con-

tents. In spite of the difficulty of quantitatively evaluating the biological potency by means of the skin test, such a comparison was undertaken and nine different tuberculin powders, whose contents of nitrogen ranged from 2.9 to 15.2 per cent, were tested. The minimum dose by weight of these powders which was just sufficient to produce the maximum skin tests varied from 0.001 to 0.0125 mg., while the nitrogen contents varied only from 0.00014 to 0.00042 mg. This shows a degree of correspondence between content of nitrogen and potency of the compounds, and speaks again for the possible identity of nitrogenous substances and potency.

In order to determine whether this nitrogen was entirely in the form of protein or also some other nitrogenous compound, amino acid analyses were made on these preparations, with the Van Slyke method and later the Thimann micro method in order to be able to analyze small quantities of material. Comparison of the data obtained upon a tuberculoprotein purified by ammonium sulfate precipitation (Sample M 9, with 15.2 per cent N and 2.8 per cent reducing substances by the Shaffer-Hartmann method), and a fraction purified by ultrafiltration, passing through an 8 per cent and held back by a 10 per cent guncotton membrane (Sample H 3, with 3.7 per cent N and 38.5 per cent reducing substances) showed the latter fraction to contain much more of its nitrogen as humin, amide, and non-basic non-amino nitrogen, less as α -amino non-basic nitrogen, and all of its basic nitrogen as arginine nitrogen. Such data could lead to the conclusion that this preparation was a histone-like body. When, however, a protein fraction with 13.7 per cent nitrogen and only 4.0 per cent reducing substances was actually isolated from this low nitrogen-containing fraction, and then analyzed, it did not appear to be a histone protein, but quite similar to the former tuberculoprotein analyzed. Therefore, the possibility, pointed out by Gortner and also by Hart and Sure in their studies of casein and other proteins, namely that the presence of carbohydrate during hydrolysis of a protein causes false results in the Van Slyke analysis, had to be considered. A hydrolysis was therefore made of the Sample M 9 tuberculoprotein to which had been added 3 times its weight of the pure polysaccharide isolated from tuberculin. Only a small increase in amide and humin nitrogen occurred and a

corresponding decrease in non-basic nitrogen. The changes, however, were too small to account for the striking results obtained on the low nitrogen-containing powders. Therefore, it would seem possible that there is still another portion of the active tuberculin nitrogen in a different form and attempts will now be made to study it.

The following table gives the maximum variations so far obtained on the purified tuberculo-protein preparations as compared with the polysaccharide-containing fractions.

Substance	Total N	Reducing substances	Per cent of total N as				
			Amide	Humin	Basic	Arginine	Non-basic
Tuberculo-protein.....	13.7-	2.5-	9.1-	1.2-	8.5-	5.8-	73.1-
	15.3	4.0	14.2	2.2	17.9	7.7	78.9
Polysaccharide-containing fractions.....	2.9-	22.5-	20.4-	1.6-	15.9-	3.8-	47.5-
	11.1	38.7	31.3	6.4	26.7	8.6	60.3
Sample M 9 + polysaccharide.....	3.5	38.3	16.5	4.3	12.5	6.2	70.4

The nitrogen distribution of the tuberculo-protein preparations was similar whether the protein had been freed from the polysaccharide by (1) ammonium sulfate precipitation, or (2) by coagulation by heat, or (3) by precipitation by trichloroacetic acid.

THE POSSIBILITY OF INTERCONVERSION OF PITUITARY HORMONES AND THE FORMATION OF DERIVED HORMONES FROM THE β -HORMONE OF THE POSTERIOR LOBE

By OLIVER KAMM, I. W. GROTE, AND L. W. ROWE

(From the Laboratories of Parke, Davis and Company, Detroit)

The α - and β -hormones of the posterior pituitary differ in certain physical properties and especially in physiological behavior. The two hormones are similar in chemical composition, in molecular weight, and in reactivities to certain reagents. Because of this chemical relationship it is reasonable to hope that before such complex compounds will be reproducible by synthesis it may be possible to convert one into the other by chemical manipulations. Preliminary attempts at interconversion have not proved successful. One of the by-products has been the production of modified

compounds still possessing physiological activity, which we have designated as "derived hormones."

Derived hormones are here defined as physiologically active compounds obtained as a result of distinct chemical changes in the molecule of the original hormone as differentiated from simple reactions like salt formation.

Separation of the α - and β -hormones from each other is not to be classed with the formation of derived hormones. In this case the activity is recovered in full amount in the separated principles. They may be again added together with the protein and obtained as a mixture identical with the original. As yet there is no evidence that more than two hormones are present. Extensive work in our laboratory has shown that the β -hormone possesses both the pressor and antidiuretic activity.

The melanophore present in varying amounts in the β -hormone fraction is probably a derived hormone. The most important derived hormone we have yet obtained has a powerful antidiuretic activity substantially free of pressor activity. In view of the low sulfur content and freedom from pressor activity, we are led to believe it a new compound.

THE MALE HORMONE

By CASIMIR FUNK AND BENJAMIN HARROW

(From Casa Biochemica, Rueil-Malmaison, France, and the Department of Chemistry, College of the City of New York, New York)

In previous publications⁴⁰ we have described the preparation and properties of extracts obtained from male urine which, when injected into castrated cocks, causes a rapid growth of combs and wattles in these animals. We now wish to report an improved and much simplified method of preparing active extracts.

As heretofore, the urine is strongly acidified and extracted with chloroform, under reflux, for some 8 hours. The chloroform portion is separated off, and the residue is heated, under reflux, for 2 hours with 20 per cent sodium hydroxide solution. The product

⁴⁰ Funk, C., and Harrow, B., *Proc. Soc. Exp. Biol. and Med.*, **26**, 325, 569 (1929); Report of The Thirteenth International Physiological Congress, 89 (1929); *Am. J. Physiol.*, **92**, 440 (1930); *Proc. Second International Sex Congress*, in press (1930).

is repeatedly extracted with ether, the ether extract is evaporated, and the residue taken up in oil.

The final product is much lighter in color and freer from impurities than earlier products.

We find it convenient to prepare products so that 1 cc. of the final material is the equivalent of 10 cock units. (We define our cock unit as that amount of extract which will cause an increase in the size of comb and wattles of 20 per cent over the initial value in the course of 10 days. For each test five capons are employed.)

1 cock unit represents the equivalent of 125 cc. of urine.

While this method involves a certain loss of active material, the relative purity of the product and the comparative ease of its preparation make it an improvement over the older methods. If desired, preparations may be made containing considerably more than the equivalent of 10 cock units per cc. of the final product.

THE OCCURRENCE OF ESTRIN IN THE FECES OF HENS

By REUBEN G. GUSTAVSON

(From the Chemical Laboratories of the University of Denver, Denver)

Juhn and Gustavson were able to demonstrate the production of the brown female breast feather in the breast of the cock as a result of injecting 250 units of estrin per day. Failure to inject the birds 1 day a week resulted in the production of a black bar in the brown feather. Since the normal hen does not show this barring effect it was concluded that the concentration of estrin in the blood of the normal hen must be high. This led to a search for the hormone in the feces of the hen. Preliminary experiments indicate that the hormone content of the feces of the hen compares favorably with that found in pregnancy urine.

DIMINUTION IN CHLORIDE MEASUREMENT AFTER DRYING BLOOD AND TISSUES

By F. WILLIAM SUNDERMAN

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania and the Ayer Clinical Laboratory, Pennsylvania Hospital, Philadelphia)

The amount of chloride recovered from blood and tissues after drying was always less than the amount recovered from blood and

tissues analyzed in the wet state. When the dried samples were kept in contact with water before $\text{AgNO}_3\text{-HNO}_3$ digestion, the quantity of chloride recovered was practically the same as that recovered from the original wet samples. Experiments suggest that fats (or fatty acids) may be responsible for the difficulty encountered.

METHODS FOR ANALYSIS OF TISSUE FOR CERTAIN INORGANIC CONSTITUENTS

By R. L. KUTZ

(From the Department of Biochemistry, McGill University,
Montreal, Canada)

Ashing of Tissue—5 gm. of dried tissue are refluxed with 25 cc. of fuming nitric acid in a 200 cc. silica Erlenmeyer flask which is ground to fit a silica reflux condenser. The tissue hydrolysate is then evaporated to dryness on a sand bath at $150\text{--}200^\circ$. The residue becomes charred, after which ashing is completed at a temperature not exceeding $300\text{--}350^\circ$ by adding 1 cc. portions of fuming nitric acid successively until the residue is white. This ash may be dissolved in dilute hydrochloric acid. It is usually necessary to reflux for a short time to dissolve phosphate which may have been converted to the meta form. Aliquots of this solution may be used for numerous determinations of inorganic constituents.

Calcium—After neutralization to methyl red the calcium is precipitated by potassium oxalate, centrifuged, and the precipitate washed with 50 per cent alcohol. The alcohol is decanted after centrifuging and the last trace removed on a water bath. The analysis is completed by the usual permanganate technique.

Potassium—This is precipitated by sodium cobaltinitrite. After the decomposition of the precipitate with perchloric acid, the perchlorates of sodium and cobalt are removed with butyl alcohol. The potassium salt is converted to the acid tartrate which is titrated with standard alkali.

Magnesium—This is precipitated as magnesium ammonium phosphate from the supernatant fluid obtained in the determination of calcium. The phosphorus content of the precipitate is determined.

Phosphorus—This is determined by the Fiske and Subbarow

method after evaporating in the presence of sulfuric acid to remove the hydrochloric acid used to dissolve the ash.

THE PSEUDOPEROXIDASE METHOD OF WU FOR THE DETERMINATION OF MINUTE AMOUNTS OF HEMOGLOBIN

BY FRANKLIN C. BING AND REGINALD W. BAKER

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland)

In a study of the development and prevention of nutritional anemia in suckling rats and mice it became essential to determine the Hb concentration of the blood without sacrificing the smaller animals. With this end in view the sensitive benzidine method of Wu⁴¹ was investigated. It has been found essential to allow the benzidine-blood-H₂O₂ mixture to digest 1 hour for full color development and, after dilution with acetic acid, to stand 8 minutes before being read in the colorimeter. In our hands strict proportionality between color development and Hb concentration has been repeatedly noted with samples that contained, after a 1 to 2000 dilution, from 0.006 to 0.100 mg. of Hb per cc., with use of a single standard containing 0.050 mg.

Accurate determinations of Hb can be made upon samples of blood measuring 0.0100 to 0.0005 cc., special micro pipettes being used. The volume taken for the determination can therefore be adapted to the size of the drop of blood available, and samples can be taken from the tails of rats and mice without the squeezing or milking procedures that tend to give erroneously high results. Precise Hb figures have been obtained with extremely small amounts of blood from anemic rats having less than 4 gm. of Hb per 100 cc. The method has been checked by parallel determinations with the O₂ capacity method of Van Slyke on normal and pathological human blood, and blood from the mouse, rabbit, and chicken. Simultaneous determinations were also made on small mice to compare the values obtained from a minute sample of tail blood with larger samples taken directly from the neck vessels. The maximum error has been within ± 4 per cent, the accuracy of measuring small samples of blood apparently being the limiting factor.

⁴¹ Wu, H., *J. Biochem.*, Japan, **2**, 189 (1923).

**AN INVESTIGATION OF THE CONDITIONS INFLUENCING THE USE
OF THE CITRIC ACID ENZYME IN CUCUMBER SEEDS AS A
MEANS OF QUANTITATIVELY DETERMINING THE
PRESENCE OF CITRIC ACID**

BY MILDRED ADAMS

(From The Mayo Clinic and The Mayo Foundation, Rochester, Minnesota)

As the result of an investigation of the dehydrogenases present in the many varieties of seeds, Thunberg⁴² has obtained a method for the determination of citric acid by means of the citric acid dehydrogenase present in cucumber seeds. Ostberg⁴³ has made a further study of this method and its use in a biological medium and has obtained by means of it considerable information as to the presence of and conditions influencing the concentration of citric acid. In an attempt to make use of this method there has been considerable difficulty due to the presence of interfering substances in the seeds generally available here. Therefore an investigation has been made to determine what factors influence this determination considering not only the influence on the rate of decolorization of the methylene blue by citric acid in the presence of the enzyme but also the influence of these factors on the interfering substances present.

Two factors found to be of considerable importance were the method for obtaining the extract from the seeds and the reaction of the enzyme extract. The influence of the concentration of methylene blue has also been further investigated. In the original method the seeds are extracted by means of a 0.05 M secondary potassium phosphate solution. It was found from this investigation that in general a water extract is preferable. Phosphate extracts are more active than water extracts in decolorizing methylene blue solutions in the absence of citric acid. A study of the influence of the reaction of the enzyme extract was made by diluting the water extract of the enzyme with phosphate mixtures varying the pH from 4.9 to 11.3. Above pH 8.5 the spontaneous decolorization of methylene blue occurs very rapidly and the results obtained were very irregular. The minimum time for the

⁴² Thunberg, T., *Biochem. Z.*, **206**, 109 (1929).

⁴³ Ostberg, O., Thesis, Physiological Institute, University of Lund, Sweden (1930).

decolorization of methylene blue in the presence of citric acid decreases somewhat in the more acid solutions but the differences between pH 6.2 and 8.3 are not great and in view of the fact that the more acid solutions enable us to decrease the influence of other substances these more acid solutions are desirable. Solutions more acid than pH 6.2 are not desirable, however, because of the increasingly long time for the minimum points and at pH 4.9 the enzyme activity under the conditions maintained here appears to be almost completely destroyed. The concentrations of methylene blue investigated were 1:50,000; 1:40,000; and 1:30,000. The more dilute methylene blue solutions permit the determination of smaller concentrations of citric acid but with the phosphate extract generally used we have been unable to obtain satisfactory results with concentrations of methylene blue less than 1:30,000. By the use of 1:50,000 methylene blue solutions and a primary water extract of the enzyme diluted with a potassium phosphate solution of pH 6.2 it is possible to detect in the methylene blue tube the presence of 0.002 to 0.003 mg. of citric acid whereas by direct phosphate extraction with a solution of pH 8.2 for extraction as originally recommended and a methylene blue solution 1:22,500 a concentration of at least 0.004 to 0.005 mg. was necessary. The concentration of methylene blue appears to have very little effect on the minimum time for decolorization.

The method as now developed consists in the use of a water extract of the seeds, dilution with a phosphate solution of pH 6.2, and a final concentration of 1 part of seeds in 15 of extract and a methylene blue solution, 1:50,000. This permits the use of urine in a primary dilution 1:25 or 1:50 when the usual amount of citric acid is present. The concentration of the interfering substances which are present in the urine is then considerably reduced and the urine curves appear to be quite characteristic of the curves obtained with pure citric acid under these same conditions.

FURTHER STUDIES ON HISTAMINASE

BY E. W. MCHENRY AND GERTRUDE GAVIN

*(From the Department of Physiology, University of Toronto,
Toronto, Canada)*

Previous work had demonstrated that a powder containing the histamine-inactivating substance (histaminase) in stable form

could be prepared from fresh beef kidney. The influence of various solvents and of hydrogen ion concentration on the yield of histaminase from this source has been investigated. Aqueous solutions can be concentrated without loss by distillation *in vacuo*. From the aqueous solution histaminase can be quantitatively precipitated with ammonium sulfate and other salts. Attempts have also been made to elucidate the nature of the histamine-histaminase reaction by measurements of the velocity constant and of the gaseous exchange.

THE ENZYMES OF A TRANSPLANTED INTESTINAL LOOP IN THE DOG

By H. B. PIERCE, E. S. NASSET, AND JOHN R. MURLIN

(From the Department of Vital Economics, The University of Rochester, Rochester, New York)

In two dogs a portion of upper jejunum was transplanted to the mammary gland, leaving the mesenteric blood supply intact.

Cannulæ were unsatisfactory for the collection of the succus entericus because of leakage. The device now in use is a loose fitting rubber tube which lies in the segment of gut and extends out to the bottom of a small beaker suspended beneath the fistula.

When the volume of juice collected is relatively large the amount of visible solid material is relatively small.

The substrates used have been 1 and 2 per cent soluble starch and sucrose solutions, 5 per cent peptone, 5 per cent casein, 2 per cent albumin, and olive oil. The starch and sucrose solutions and the olive oil were neutral in reaction; the remaining substrates were adjusted to pH 7.6.

The sucrose and amylase activity were estimated by determination of the amount of reducing sugars by the Bertrand method. Protein and peptone hydrolysis were determined by formol titration. The lipolytic activity was measured by titrating the fatty acids liberated from olive oil by alcoholic KOH.

Thus far the study has revealed the presence of sucrase, amylase, lipase, and ereptase.

STUDIES ON THE TRYPSINOGEN-ENTEROKINASE SYSTEM

By ROBERT W. BATES

(From the Department of Physiological Chemistry and Pharmacology, the University of Chicago, Chicago)

These studies have been made with casein as a substrate using the refractometer method for measuring the amount of hydrolysis by the trypsin. Trypsin-free trypsinogen and enterokinase were used.

The course of activation of trypsinogen with variable time and at several enterokinase concentrations has been followed. The effects of KCl and pH upon such curves have also been considered. Maximum activation occurs at pH 6.0 but the time of this maximum varies with the concentration of enterokinase, pH, and ionic concentration. Rapid destruction of the trypsin after its formation is shown. These results throw much doubt upon all work in which activation is a variable.

It has been found that activation takes place during the digestion. Upon the basis of this finding a method for the assay of enterokinase has been worked out, in which the activation occurring in the presence of the casein substrate during digestion is the only activation. This is an amount easily reproduced because it is free from the errors of variable time, pH, and salt concentrations.

STUDIES OF PEPTIC ACTIVITY. THE LIBERATION OF ARGININE AND TYROSINE COMPLEXES IN PEPTIC PROTEOLYSIS

By VIRGINIA TORBET AND H. C. BRADLEY

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison)

In a peptic digestion of casein, tyrosine was unmasked very rapidly (50 per cent in 1 day, 70 per cent in 5 days). In a 30 day digest nearly 100 per cent of the tyrosine was rendered active to the Folin-Looney phenol reagent. The general course of hydrolysis as measured by Van Slyke amino N and Sorensen titration proceeds much more slowly, and opens about 30 per cent of the possible unions. The tyrosine curve so nearly coincides with the fragmentation of the casein into peptides not precipitated by

trichloroacetic acid, as to warrant the assumption that cleavage at tyrosine unions is one of the points most labile to pepsin.

Hunter showed that two-thirds of the total arginine is unmasked by trypsin in both casein and gelatin. Pepsin liberates arginine very slowly from both gelatin and casein. At the end of 12 days about 15 to 20 per cent is unmasked, the reaction still proceeding slowly.

Cystine and the carbohydrate of casein are slowly rendered soluble, but never completely so. The insoluble residue which regularly remains after prolonged peptic action on casein is fairly rich in carbohydrate (Molisch test) and contains about 0.4 per cent of cystine. It is a peptide, and contains about 1 per cent of tyrosine by the Folin-Looney test, and about 10 per cent of the original casein nitrogen remains in this residue.

SOME PROPERTIES OF YEAST INVERTASE

BY J. M. NELSON AND A. H. PALMER

(From the Department of Chemistry, Columbia University, New York)

The paper deals with the effect of neutral salts, alcohol, and high concentrations of sucrose on the velocity of enzymatic inversion of sucrose from the point of view of analogies drawn from the field of colloidal phenomena.

It has been found experimentally that the degree of retardation of the velocity of hydrolysis, due to the presence of any one of these classes of substances, is related to hydrogen ion activity. Both alcohol and high concentrations of sucrose exert maximum retardations at what may be considered as the isoelectric point of the enzyme, while the retardation due to neutral salt is at a minimum at that point. A comparison of a series of neutral salts has shown that the retardations due to the presence of equivalent amounts of these salts are of the same order of magnitude at the isoelectric point, while if this comparison is made on the alkaline side not only are the absolute retardations greatly increased but the Hofmeister series is evident as well, the order of increasing retardations being $\text{LiCl} \rightarrow \text{NaCl} \rightarrow \text{KCl} \rightarrow \text{KBr} \rightarrow \text{KI} \rightarrow \text{BaCl}_2$.

BLOOD HEMOGLOBIN IN THYROID ENLARGEMENT*

By ROE E. REMINGTON

(From the Laboratory of the South Carolina Food Research Commission and the Department of Nutrition of the Medical College of the State of South Carolina, Charleston)

South Carolina is recognized as a relatively non-goitrous area. This is based on examinations of 18,000 school children, and inferentially on analyses which show a relatively high iodine content in plants grown in this region. Basal metabolism determinations on normal people in South Carolina, however, show values lower than the accepted standards. Three determinations were made on different days, on each of 93 white student nurses at Charleston. When the lowest value for each subject is used, the average is 10.4 per cent lower than the Aub-DuBois standard. On these same women, the average red blood cell count was 5.01 millions, hemoglobin 12.4 gm. per 100 cc.

Similar values for seventeen negro girls with marked thyroid enlargement (high school students, ages 15 to 22) were — 14.0 for lowest values, and 4.04 millions and 9.3 gm. of hemoglobin.

On the assumption that the main source of iodine for these people is in leafy plants, and that the development of goiter in the negro girls is due to an improper diet, lacking in vegetables, the anemia can be explained by a deficiency in mineral elements necessary in blood formation, which are found abundantly in the same foods. It might, of course, be explained as a concurrent manifestation of the thyroid condition itself, although hypothyroidism was not marked.

Enlarged and congested thyroids have been produced in rats by the Steenbock rachitic diet (high Ca, low P), and by a diet perfect in all known respects except for a deficiency of iodine, and in rabbits by cabbage. Blood changes paralleling these thyroid changes are being studied.

* Presented before the American Society for Experimental Pathology.

**INORGANIC SALT METABOLISM DURING INVOLUTION OF
SIMPLE GOITER***

BY EMIL J. BAUMANN

(From the Division of Laboratories, Montefiore Hospital, New York)

The influence of varying states of activity of the thyroid gland upon mineral metabolism was studied in three rabbits. The rabbits were fed on a diet consisting chiefly of cabbage (which will cause simple goiter) until clinically palpable thyroids were developed, after which they were placed in metabolism cages and the mineral intake and output determined for two 3 day fore periods. They were then injected intraperitoneally with a few mg. of iodine to involute the thyroid (this amount of iodine will restore the gland to the normal state). The study of the mineral intake and output (Na, K, Ca, Mg, Cl, P, S) was continued for 15 to 21 days on the cabbage diet, during which time the state of the thyroid returned to normal and finally back again to the hyperplasia of simple goiter.

The most striking results were a retention of calcium, magnesium, and phosphorus while the animals were in a hypothyroid state. Involution of the gland with iodine, thus changing it to the resting stage, practically restored the calcium, magnesium, and phosphorus balance.

HORMONES IN CANCER**III. EFFECT OF GLANDULAR EXTIRPATION***

BY FRITZ BISCHOFF, L. C. MAXWELL, AND H. J. ULLMANN

(From the Department of Cancer Research, Santa Barbara Cottage Hospital, Santa Barbara)

The survival of rats after adrenalectomy was sufficiently long so that the rate of growth of a transplantable sarcoma and carcinoma inoculated after adrenalectomy could be followed. No significant increase in tumor growth as compared with controls could be demonstrated. Castration (both male and female) had no effect upon the rate of growth of the sarcoma, and splenectomy no effect upon the carcinoma. In the case of the sarcoma, splenectomy increased the rate of tumor growth by 3 times the standard

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deviation of the mean. When sublethal doses of x-ray were applied to the head of the rat, the rate of growth of the tumor was markedly retarded during the period when the growth of the rats ceased. Other treatment (synthalin, thyroxine, etc.) also stops the growth of the rat but does not affect the rate of tumor growth. Raying the head was designed to inhibit or destroy the pituitary body.

PLASMA FATS IN SOME CASES OF MENTAL DEPRESSION*

By ELEANOR M. HILL, C. N. H. LONG, AND DAVID SLIGHT

(From the University Clinic, Department of Medicine, McGill University and Royal Victoria Hospital, Montreal, Canada)

The following is a study of the plasma fats in a series of twelve cases of mental depression as compared with ten normal subjects (Table I). Estimations were made in the fasting state and 4 hours after a meal containing 70 gm. of fat.

TABLE I
Plasma Fat Analysis

	Total fatty acids (tripalmitin per 100 cc. plasma)	Iodine absorbed per 100 cc. plasma	Iodine No.	Cholesterol per 100 cc. plasma
Normals (10 cases)				
Fasting	gm.	gm.		mg.
Average.....	0.364	0.386	112	204
Range.....	0.240-0.565	0.269-0.486	79-182	107-286
4 hrs. after fat meal				
Average.....	0.447	0.434	103	209
Range.....	0.268-0.619	0.276-0.544	76-202	110-278
Mental depression (12 cases)				
Fasting				
Average.....	0.565	0.400	78	190
Range.....	0.323-0.834	0.236-0.532	45-155	146-250
4 hrs. after fat meal				
Average.....	0.847	0.428	57	180
Range.....	0.484-1.910	0.230-0.610	31-98	133-229

* Presented before the American Society for Experimental Pathology.

The total fats were estimated by the method of Stewart and White as modified by Nicholls and Perlzweig.⁴⁴ The modification of the Hanus method as described by Gibson and Howard⁴⁵ was used to determine the iodine number. Cholesterol determinations were made according to the method of Myers and Wardell on the fresh plasma.

CONCLUSIONS

1. The total fatty acids of the plasma in certain cases of mental depression are higher than normal, both in the fasting state and after a fat meal.

2. This difference appears to be due to the greater amount of saturated acids in the abnormal cases.

3. No significant changes were noted in regard to cholesterol.

STUDIES IN EXPERIMENTAL KIDNEY INSUFFICIENCY*

By ALFRED CHANUTIN, EUGENE B. FERRIS, AND J. EDWIN WOOD

(From the Laboratory of Physiological Chemistry, University of Virginia, University)

After removal of about 80 to 85 per cent of the total kidney tissue in the white rat by a two stage operation, a study was made of the kidney function, blood non-protein nitrogen, blood pressure, and heart and kidney weights. Many of these animals die "acutely" but the majority may live for varying periods up to 5 months.

Animals (fed a 20 per cent protein diet) surviving this operation for 2 or more months show a definite picture. The majority of the nephritic rats have a high blood pressure as demonstrated by direct cannulization of the carotid artery. Many of these animals may have a normal or subnormal blood pressure at the time they were killed. Regardless of blood pressure, however, there is invariably a definite cardiac hypertrophy, as measured by the heart weight: surface area ratio, accompanied by a dilatation of the carotid artery. The non-protein nitrogen may remain normal for a long time and then rise markedly or there may be a continued

⁴⁴ Nicholls, E. G., and Perlzweig, W. A., *J. Clin. Inv.*, **5**, 195 (1927-28).

⁴⁵ Gibson, R. B., and Howard, C. P., *Arch. Int. Med.*, **32**, 1 (1923).

* Presented before the American Society for Experimental Pathology.

high non-protein nitrogen for a period of months. When these rats become sick, the non-protein nitrogen is high varying between 88 and 312 mg. per 100 cc. of blood. A progressively increasing polyuria with a low specific gravity is seen. Hyaline and granular casts are noted. The protein output in the urine is very small shortly after the operation but gradually increases over a period of several months until there is a very marked albuminuria. No edema has ever been noted.

Rats dying within a month after the operation show no signs of increased blood pressure, cardiac hypertrophy, or arterial dilatation. In fact the blood pressure tends to be subnormal. The blood non-protein nitrogen is very high varying between 111 and 454 mg.

Rats fed a high protein diet (75 per cent) show the same type of results noted in the above animals. The kidney is more markedly hypertrophied in this group. It has been noted that there is a larger number of acutely ill rats fed a 75 per cent protein diet than there is in the case of animals receiving 20 per cent protein, high urea, or creatine diets.

The so called acute type of kidney shows a rather marked dilatation of the distal convoluted tubules with a slight dilatation of the proximal convoluted tubules. No very striking degeneration occurs in either. The glomeruli show slight hypertrophy. Only an occasional glomerulus shows atrophy, and hyaline degeneration is rare or absent. The reticular tissue between the tubules has increased moderately and many intertubular blood spaces have been eliminated. Occasional groups of leucocytes appear in the interstitial tissue.

In the chronic type of kidney there is a well marked dilatation of the proximal convoluted tubules. The distal convoluted tubules show moderate dilatation, and in addition definite hyaline droplet degeneration. The intertubular blood spaces have largely disappeared and these spaces are now greatly increased with reticular tissue (argyrophilic fibers). There is no apparent thickening of the basement membrane of the tubules. The number of glomeruli are decreased but those remaining show hypertrophy. Hyaline degeneration of the glomeruli occurs frequently. There is no thickening of the glomerular capsule or crescent formation.

Marked thickening of the renal capsule takes place. The sharp

increase in interstitial reticular tissue and the dilated tubules filled with fluid largely account for the increase in weight of the kidney stump. Some of the dilated tubules in the older hypertrophied kidney stumps are well filled with hyaline material.

**NEPHRITIS IN UNILATERALLY NEPHRECTOMIZED WHITE RATS
LIVING UPON HIGH PROTEIN DIETS***

By N. R. BLATHERWICK, E. M. MEDLAR, J. M. CONNOLLY, AND
PHOEBE J. BRADSHAW

*(From the Biochemical Laboratory and the Hegan Memorial Laboratory of
the Metropolitan Life Insurance Company, New York)*

The right kidney of young albino rats was removed when the animals weighed about 100 gm., and the rats were placed immediately upon the experimental diets. Six diets have been used, as follows: the control diet consisting of wheat, dried milk, and dried beef muscle, with a content of about 24 per cent protein; Muscle Diet I containing 75 per cent dried beef muscle and with a content of about 69 per cent protein; Liver Diet I containing 75 per cent dried beef liver and with a content of about 48 per cent protein and having a Ca:P ratio of 0.27; Liver Diet II was similar to Liver Diet I except for an improved Ca:P ratio of 1.04; Liver Diet III was similar to Liver Diet II except for the addition of enough sodium bicarbonate to cause the production of a neutral urine; and Liver Diet IV was the same as Liver Diet II except that dried liver residue⁴⁶ remaining after the removal of the pernicious anemia fraction replaced dried whole liver. All of the above diets were supplemented with appropriate additions of yeast, cod liver oil, lard, and salts. Lettuce and carrots were fed twice a week. All of the rats were reared in our laboratory on the control diet.

Albumin and casts appeared in the urine of the rats eating the muscle and liver diets at about 3 months. The urine of the control animals showed nothing abnormal for the 1st year and has rarely indicated the severe injury produced by the other diets. Most of the casts were granular and contained fat. Pigmented

* Presented before the American Society for Experimental Pathology.

⁴⁶ We are indebted to Eli Lilly and Company for the supplies of dried liver residue and of sodium amylal used in these experiments.

casts were frequently observed. Cylindroids occurred in greater numbers than did casts.* Albumin was excreted in large amounts as shown by common values of about 4 per cent.

The rats were allowed to live until it was evident that death would soon result. The records show that six of the eight rats on the control diet were alive at 553 days, three of nine on Muscle Diet I were alive at 566 days, one of thirteen on Liver Diet I was alive at 585 days, three of twelve on Liver Diet II were alive at 580 days, three of twelve on Liver Diet III were alive at 392 days, and nine of twelve on Liver Diet IV were alive at 392 days. Male rats were more easily injured by the diets than were the females.

These nephritic animals show marked retention of urea, an inverted albumin:globulin ratio in the blood plasma, and high values for plasma cholesterol.

The kidneys at the end of the experiments show marked hypertrophy, varying from 2 to 3 times the weight of two normal kidneys. They are often very edematous. They are of a mottled red and greyish-red color. The surface has an irregular, pitted appearance.

Microscopical examination shows a typical chronic glomerular nephritis very similar to that seen in man. The lesions vary from the involvement of a small part of the glomerulus to complete sclerosis of the structure. This sclerosis includes the obliteration of the capsular space. The convoluted tubules commonly show marked dilatation and are often filled with large hyaline casts. In places there is marked hyperplasia of the tubular epithelium. Some parts of the convoluted tubule epithelium show marked accumulation of dark brownish pigment. The collecting tubules appear normal.

EFFECT OF ESTRIN INJECTIONS ON THE ANTERIOR LOBE*

By F. E. D'AMOUR

(From the Department of Zoology, University of Denver, Denver)

Moore and Price have advanced the theory that gonadal hormones inhibit the production of gonad-stimulating secretions from the anterior lobe. We wished to learn whether this inhibition

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also extended to the growth-promoting hormone known to be produced by this gland.

Twenty-five young rats were injected daily with 20 units of a very pure estrin preparation and their curves compared with curves from non-injected animals. It was found that they grew on an average of 20 to 25 per cent more slowly than the controls.

In a preliminary experiment it was observed that the sex glands were markedly inhibited. In the present study it was found that the weight of the control ovaries was 2 to 3 times as great as those of the injected animals and the weight of the testes 3 to 4 times as great. Histological studies also show marked changes.

Work is in progress to learn whether this growth inhibition is due primarily to a repression of the hypophysis or to some other cause.

BLOOD ALCOHOL ESTIMATION AND ITS RELATION TO INTOXICATION*

By R. G. TURNER

(From the Department of Medical Research, Detroit College of Medicine and Surgery, Detroit)

The investigation includes a study of the estimation of alcohol in aqueous solutions and in blood by various methods based on its oxidation with sulfuric acid and bichromate. Eleven blood alcohol absorption curves on dogs following alcohol ingestion are presented.

The alcohol determinations were made by a modification of the Nicloux method. A modification of the Shaffer method for the estimation of ether in blood has been applied to the determination of alcohol as a comparison for the Nicloux method.

Results show that the Nicloux method is applicable to the detection of blood alcohol in concentrations of 0.2 to 0.5 per cent with a range of error from 2 to 5 per cent. In lower concentrations (0.1 per cent or less) the error may reach as high as 15 per cent.

Intoxication in dogs is definite when the blood alcohol reaches 0.25 per cent, marked at 0.3 per cent, and alcoholic stupor and coma result at 0.4 to 0.5 per cent. The intensity of intoxication

* Presented before the American Society for Pharmacology and Experimental Therapeutics.

is less when ingestion takes place during or after a meal. Ingestion of dilute solutions (10 per cent) produces a less intense intoxication than strong solutions (40 per cent).

TEMPERATURE AND MUSCULAR ACTIVITY

BY D. B. DILL AND H. T. EDWARDS

(From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston)

The effects of external temperature and humidity on performance have been studied in the case of several individuals. Each subject did the same work on the bicycle ergometer with (a) an external temperature of $12 \pm 1^\circ$ and (b) an external temperature of $34 \pm 1^\circ$ and a relative humidity of 45 ± 5 per cent in each case. The work done involved an oxygen consumption of 1.9 ± 0.5 liters per minute and with a moderate or small oxygen debt. Rectal temperature was observed frequently, a thermocouple being used, and heart rate was recorded continuously with a cardiometer. Observations on the metabolism indicated that work is carried on with the same mechanical efficiency and with the same fuels under these extreme conditions.

The rate of increase of body temperature as work is carried on is nearly constant for the first few minutes. Then a constant temperature is reached if conditions for heat dissipation are favorable; otherwise body temperature rises until exhaustion intervenes.

The heart rate increases with external temperature even when internal temperature is the same. Its output may remain constant or increase slightly. Consequently its output per beat must diminish with increasing external temperature. Blood supply to the skin and inactive muscles increases and to active muscles diminishes with increasing external temperature.

In our experiments four of the five subjects quit from exhaustion at the high temperature when doing work which they carried on easily at a low temperature. Yet there was no considerable lactic acid accumulation in the body as a whole, no exhaustion of fuel reserves, and a large unused reserve of pulmonary ventilation. The most probable hypothesis is that the heart muscle itself had become exhausted.

GASTROINTESTINAL TEMPERATURE STUDIES*

By HARRY M. EBERHARD, ROWLAND RICKETTS, CHARLES L. W. RIEGER, AND JOSEPH S. HEPBURN

(From the Hahnemann Medical College, Philadelphia)

The research includes a study of: (1) the temperature of the human stomach, and the influence thereon of ice water, ice-cream, hot coffee, and physiotherapeutic agents (hot water bag, ice bag, diathermy, infra-red lamp, electric pad); (2) the effect of ice water on the gastric secretion and emptying time; and (3) the temperature of the human duodenum and the influence thereon of physiotherapeutic agents.

THE IODINE CONTENT OF BLOOD IN CERTAIN PATHOLOGICAL CONDITIONS

By R. G. TURNER AND C. W. MATTHEWS

(From the Department of Medical Research, Detroit College of Medicine and Surgery, Detroit)

The iodine content of the blood has been determined on 50 diabetic cases, ten hyperthyroid cases, five hypothyroid cases, and on ten various other pathological cases.

The results show a marked elevation of the blood iodine in the first stages of diabetes. A definite increase is found in hyperthyroidism followed by a decrease after operation. Hypothyroid cases show a normal iodine content. Cases of cardiac decompensation, catarrhal jaundice, and lues show no variation from the normal.

THE CONTENT AND NATURE OF THE HIGHLY UNSATURATED FATTY ACIDS OF THE HUMAN BRAIN

By J. B. BROWN

(From the Laboratory of Physiological Chemistry, the Ohio State University, Columbus)

Fresh and preserved specimens of human brains have been subjected to aqueous alkaline hydrolysis, the fatty acids have been recovered, and their solutions in ether have been brominated. The purified ether-insoluble bromides contained from 65 to 67 per cent

* Presented before the American Physiological Society.

bromine. The yields were not essentially different from those obtained from sheep, beef, and pork brains. Individual brains showed wide variations in yield of bromides.

**THE RELATIONSHIP BETWEEN INCREASE IN BODY WEIGHT AND
HEMOGLOBIN AFTER IRON THERAPY IN NUTRITIONAL
ANEMIA OF THE RAT**

By HOWARD H. BEARD

*(From the Department of Biochemistry, School of Medicine, Western Reserve
University, Cleveland)*

The average increase in body weight for the first 3 weeks after Fe therapy in young rats suffering from nutritional anemia has been compared with that secured when other elements were added to the Fe.

The results show that growth was better when optimum doses of other elements were added to Fe, in the order named, Cu, Ti, Ni, Se, As, Ge, Zn, Mn, Cr, V, Rb, and Hg. The addition of these elements stimulated growth as well as blood regeneration.

Doses of these elements which had no influence upon blood regeneration greater than Fe alone, with three exceptions (Cu, Zn, and Mg), retarded growth in the order named, V, Hg, Cr, Rb, Se, Mn, As, and Ni.

A statistical analysis has been made of the relation of body weight to hemoglobin increase after the feeding of 0.5 mg. of Fe for periods of 3 and 6 weeks. The results obtained showed the following.

$$\begin{aligned}\text{Correlation coefficient, } r &= 0.49 \pm 0.06 \text{ (3 weeks)} \\ &= 0.29 \pm 0.01 \text{ (6 ")}\end{aligned}$$

This shows that there was a much greater relation between body weight and hemoglobin increase at the end of 3 weeks than at the end of 6 weeks.

The prediction equation obtained with the first correlation coefficient was $y = 2.61841x + 4.56170$, where y = increase in body weight and x the increase in hemoglobin. Substituting the average increases in hemoglobin of each group of animals in this equation it was shown that there was a linear relationship between these two variables.

THE DISTRIBUTION OF CHLORIDE AND BICARBONATE BETWEEN
PLASMA AND CELLS IN THE BLOOD OF VARIOUS PATHO-
LOGICAL SUBJECTS

By EDWARD MUNTWYLER, EMBREE R. ROSE, AND
VICTOR C. MYERS

(From the Department of Biochemistry, School of Medicine, Western Reserve
University, Cleveland)

The distribution ratios $[\text{cell Cl}]:[\text{plasma Cl}]$ and $[\text{cell BHCO}_3]:[\text{plasma BHCO}_3]$ have been determined in a large number of pathological blood specimens. The study was made along two lines. Distribution ratios were determined on venous blood taken under oil without stasis, and hence under existing CO_2 tensions, by determining the concentrations of chloride and bicarbonate in the cells indirectly from whole blood and plasma analyses. Distribution ratios were also determined on bloods at equilibrium after equilibration with varying tensions of CO_2 at complete oxygenation, the chloride concentrations of the cells being determined by direct analysis and the bicarbonate by indirect.

With the first group of experiments on venous content there was a marked similarity in the distribution ratios at a given pH level (when corrected to complete oxygenation) irrespective of the type of condition. With an average pH of 7.42 the average r_{Cl} was 0.670 and the average r_{HCO_3} , 0.753, giving an $r_{\text{Cl}}:r_{\text{HCO}_3}$ ratio of 0.89. Bloods with markedly lowered pH values had distribution ratios approaching unity. A severe case of bichloride poisoning having a plasma pH of 7.07 showed the maximum observed r_{Cl} of 1.029 and r_{HCO_3} of 1.269. Two cases of chronic nephritis with plasma pH values of 7.28 and 7.10 respectively had r_{HCO_3} values above unity.

The ratios obtained after equilibration with known tensions of CO_2 at complete oxygenation were plotted against pH as were also the average distribution ratios for a given pH obtained from the venous bloods. The ratios varied with the pH in agreement with the views outlined by Van Slyke, Wu, and McLean.⁴⁷ The data also give support to the conclusions of Hastings, Sendroy, McIntosh, and Van Slyke⁴⁸ that the r variations at a given plasma

⁴⁷ Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, **56**, 765 (1923).

⁴⁸ Hastings, A. B., Sendroy, J., Jr., McIntosh, J. F., and Van Slyke, D. D., *J. Biol. Chem.*, **79**, 193 (1928).

pH show no greater variation in pathological conditions than in normals presumably because of the constancy of the hemoglobin concentration in the cells and the total base of the serum, which are the most important in fixing the value of r .

DERIVATIVES OF THIOPYRUVIC ACID IN CONNECTION WITH A DIET DEFICIENT IN CYSTINE

By RICHARD J. BLOCK AND RICHARD W. JACKSON

(*From the Laboratory of Physiological Chemistry, Yale University, New Haven*)

Various experiments indicate that the metabolism of amino acids is closely related to that of the corresponding pyruvic acids. In view of investigations showing that α -dihydroxy- β -dithiopropionic acid, certain other disulfide acids, and taurine are incapable of replacing cystine in the diet the analogous pyruvic acid becomes clothed with considerable interest. However, since thiopyruvic acid does not appear to be a stable molecule, the authors have used in its stead some of its derivatives from which the pyruvic acid might possibly be released in the body. Diethyl-6-oxypyrimidine-2-oxalthiolglycollate, ethyl-6-oxypyrimidine-2-thiolpyruvate, and the condensation products (non-crystalline) of potassium ethyl xanthate with ethyl- β -bromopyruvate, and of potassium ethyl xanthate with β -bromopyruvic acid were prepared. None of these compounds has thus far been found to cause any weight increment in rats subsisting on diets deficient in cystine. This is a preliminary report.

A SIMPLIFICATION OF THE OKEY METHOD FOR THE DETERMINATION OF CHOLESTEROL

By MARY E. TURNER

(*From the Biochemical Laboratory, State University of Iowa, Iowa City*)

The Okey method for the micro determination of cholesterol by oxidation of the digitonide was modified for more general use. The digitonide was precipitated in a 15 cc. centrifuge tube, washed free from interfering substances with ether and water, the supernatant liquids being removed by centrifugation at high speed. The intricate procedure of filtration in the original method was thus eliminated without too great a sacrifice of accuracy. Recovery figures were 2 to 4 per cent high on free cholesterol.

THE UNSAPONIFIABLE CONSTITUENTS OF THE LIVER LIPIDS

BY F. C. FREYTAG AND H. GREGG SMITH

(From the Biochemical Laboratory, State University of Iowa, Iowa City)

In the course of a study of the composition of the unsaponifiable lipids of beef liver, fractional crystallization from methyl alcohol, acetone, and petroleum ether yielded about 50 per cent of the material as four separate fractions of cholesterol, the remainder being non-crystalline. The physical constants of the cholesterol fractions have been found to be sufficiently different from each other to warrant further study. Attempts are also being made to characterize the non-crystalline portion.

VITAMIN A CONTENT OF RETINAL AND CHOROIDAL TISSUEBY ARTHUR H. SMITH, ARTHUR M. YUDKIN, MAX KRISS, AND
HARRY M. ZIMMERMAN*(From the Laboratory of Physiological Chemistry, the Department of Surgery, and the Department of Pathology, Yale University, School of Medicine, New Haven)*

The occurrence of vitamin A in the retina of calf eyes was reported in 1929 by Holm.⁴⁹ Using the fresh tissue as the source of this factor, he found that 750 mg. per day were required for normal growth of rats, though 50 mg. sufficed to cure ophthalmia. Brain tissue exhibited little potency. Holm's basal ration contained no recognized source of vitamin D nor were the animals treated with ultra-violet light. In the present study, 400 mg. of irradiated dried yeast were given apart from the basal vitamin A-deficient diet as a source of both the vitamin B complex and the antirachitic factor.

The retinal tissue was procured from pig eyes and was dissected as free as possible from the black choroid; roughly 1 gm. was obtained from 100 eyes. After being washed with water several times, the tissue was rapidly frozen with solid CO₂ and transferred to a vacuum desiccator. The resulting light tan, friable powder was preserved in a brown bottle, in an atmosphere of CO₂ in the refrigerator. It contained 12 per cent nitrogen, 6 per cent moisture, 20 per cent ether extract, and 18 per cent hot absolute alcohol extract.

⁴⁹ Holm, E., *Acta Ophthalm.*, 7, 146 (1929).

In the negative control and curative experiments, ophthalmia and cessation of growth occurred upon the basal diet alone in 23 to 29 days. Dried retinal tissue was then given. Relief of the eye condition, resumption of growth, and incidence of abscesses in lingual glands and ear, and renal and vesicular calculi were the criteria upon which the effectiveness of treatment was based. 50 mg. of retinal tissue were highly potent; 30 mg. were almost as effective, while 20 mg. were only partially so. However, even 10 mg. brought about considerable degree of relief of ophthalmia. Choroidal tissue in quantity of 50 mg., on the other hand, was ineffective as a source of vitamin A under these conditions. When the animals were not subjected to the initial depletion of their stores of vitamin A, 20 mg. of dried retina per day permitted as rapid growth as 100 mg. of butter fat and no abnormalities characteristic of vitamin A deficiency were detected.

In the curative experiments there was found histologically a marked proliferation of the fibroblastic elements of the choroid accompanied by a decidedly increased vascularity of this structure. Numerous mitotic figures were found here as evidence of a proliferative reaction. However, rats cured with butter fat showed a similar picture, but control animals invariably failed to show this.

The blue color with arsenic trichloride was given very distinctly by the ether extract of retina but not at all by the alcohol extract. If this reaction is indicative of the presence of vitamin A, retinal tissue can be considered one of the most potent known sources of this factor.

THE SPECIFIC DYNAMIC RESPONSE OF INDIVIDUALS SUFFERING FROM DISTURBANCES OF THE HYPOPHYSIS

By MARGARET WOODWELL JOHNSTON

(From the Department of Internal Medicine, Medical School, University of Michigan, Ann Arbor)

It has been stated that individuals suffering from diseases of the hypophysis do not exhibit the usual increase in heat production following the ingestion of a protein meal. Meager information contained in the publications leaves one in doubt with regard to the diagnosis.

We have studied a series of patients who presented definite evidence of pituitary disease, as shown by (1) erosion of the sella turcica and the existence of optic atrophy, (2) operation, or (3) necropsy. The basal metabolic rate of these individuals was determined by the Tissot method until satisfactory results were obtained. Then 300 gm. of ground lean sirloin steak were fed, and the heat production determined at intervals of approximately 1 hour for 3 or 4 hours. In all cases a definite increase occurred. This response varied, however, both with regard to time and magnitude. Increases from 14 to 23 per cent were observed. The maximal increase was obtained as early as 1 hour, and as late as 4 hours after the ingestion of the protein.

**THE EFFECT OF THE CALCIUM AND PHOSPHORUS CONTENT OF
THE DIET AND OF VITAMIN D UPON RESPONSE TO PARA-
THYROID EXTRACT**

BY AGNES FAY MORGAN AND E. ALTA GARRISON

*(From the Laboratory of Household Science, University of California,
Berkeley)*

Young dogs were fed, from weaning, synthetic diets of varying calcium and phosphorus content with and without the addition of cod liver oil or irradiated ergosterol. On all diets the rise in serum calcium resulting from the injection of parathyroid extract was much less in the vitamin D-free animals than in the controls.⁵⁰ On the low calcium-high phosphorus diet tetany, lameness, and terminal paralysis occurred in nearly all animals and the rise in serum calcium occurred only after the first injection of parathyroid extract unless the dosage was greatly increased. On high calcium-low phosphorus and normal diets the rise in serum calcium occurred regularly after each injection in the animals receiving vitamin D. Only small and irregular changes in serum inorganic phosphorus followed the parathyroid dosage on all diets. This is taken to mean that the source of the increased serum calcium is the food or an exceptionally mobile calcium reserve rather than the permanent bony tissues. The vitamin D-free and low calcium-fed animals appear to be alike in being entirely lacking in, or easily stripped of, this reserve with resulting failure to show the characteristic rise in serum calcium.

⁵⁰ Morgan, A. F., and Garrison, E. A., *J. Biol. Chem.*, **85**, 687 (1929-30).

EFFECT OF SULFHYDRYL COMPOUNDS ON THE RATE OF REGENERATION IN PODARKE OBSCURA

By SERGIUS MORGULIS AND DAVID E. GREEN

(From the Department of Biochemistry, University of Nebraska, Omaha)

The regeneration of the polychaete worm, *Podarke obscura*, was studied under the influence of a number of sulfhydryl compounds. The sulfhydryl compound was added every day to the sea water in which the animals were kept, and the SH concentration checked up by means of iodine titration. In none of the experiments was there any evidence of a stimulation of the regenerative process, which is strictly a process of cell multiplication. On the contrary, in concentrations just above the ineffective dose there has been either retardation of the regenerative process or signs of marked intoxication. The latter increased with concentration of the SH leading to cytolysis and complete disintegration of the organisms when the concentration became sufficiently strong.

THE ANTIDIURETIC ACTIVITY OF POSTERIOR PITUITARY EXTRACTS**II. A COMPARATIVE QUANTITATIVE STUDY OF THE EFFECT UPON MICE AND MEN**

By I. W. GROTE, EDWARD G. JONES, AND OLIVER KAMM

(From the Laboratories of Parke, Davis and Company, Detroit)

The most accurate method of testing and assaying posterior pituitary products for their antidiuretic potency has been based upon experiments on the human subject. Different individuals differ considerably in their sensitivity but responses to equivalent repeated doses in the same individual are fairly constant.

The mouse method recently proposed by Gibbs⁶¹ has been studied and perfected. Since a large group of animals can be studied simultaneously, the method is susceptible of greater accuracy than the test on one or two human subjects.

A unit is proposed as follows: The minimum amount of extract which, when administered subcutaneously, will entirely stop secretion of urine during the final 3 hours of a 5 hour test period in eight mice out of ten, which have received 1 cc. of water intraperi-

⁶¹ Gibbs, O. S., *J. Pharmacol. and Exp. Therap.*, **40**, 129 (1930).

toneally, is equivalent to 1 mouse unit. The average amount of urine per animal during the entire 5 hour period should not exceed 400 mg. and will usually lie between 275 and 375 mg.

In our study, one human subject was found to be far more susceptible than a mouse to an equal dosage of pituitary extract, whereas others required several mouse units in order to induce an antidiuretic effect lasting several hours.

**THE METABOLIC RATE AND RESPIRATORY QUOTIENTS OF RATS
ON A FAT-DEFICIENT DIET⁵²**

By LAURENCE G. WESSON AND GEORGE O. BURR

THE COMPARATIVE INFLUENCE OF VARIOUS FATS, AND OF DIFFERENT AMOUNTS OF THE SAME FAT, ON THE DEGREE OF UNSATURATION OF THE TISSUE PHOSPHOLIPIDS⁵³

By ROBERT GORDON SINCLAIR

THEELOL. PREPARATION, ASSAY, AND CHARACTERIZATION⁵⁴

By EDWARD A. DOISY, JACK M. CURTIS, LOUIS LEVIN, AND
SIDNEY A. THAYER

**A COMPARATIVE METHOD FOR THE DETERMINATION OF PEPTIC
ACTIVITY**

By JAMES B. SUMNER AND DAVID B. HAND

**OBSERVATIONS ON THE CAUSATION AND TREATMENT OF
DIABETIC COMA**

By E. C. DODDS

**FURTHER CONTRIBUTIONS TO THE CHEMICAL COMPOSITION
OF INSULIN**

By H. JENSEN, E. M. K. GEILING, AND A. DE LAWDER

FURTHER STUDIES OF THE CHEMISTRY OF TOAD POISONS

By H. JENSEN AND K. K. CHEN

⁵² Wesson, L. G., and Burr, G. O., *J. Biol. Chem.*, **91**, 525 (1931).

⁵³ Sinclair, R. G., *J. Biol. Chem.*, **92**, (1931) in press.

⁵⁴ Doisy, E. A., and Thayer, S. A., *J. Biol. Chem.*, **91**, 641 (1931). Curtis, J. M., and Doisy, E. A., *J. Biol. Chem.*, **91**, 647 (1931). Thayer, S. A., Levin, L., and Doisy, E. A., *J. Biol. Chem.*, **91**, 655 (1931).

STUDIES ON THE PURIFICATION OF PEPSIN

By M. R. FREELAND

**A METHOD FOR THE DETERMINATION OF HEXOSEPHOSPHATE
IN MUSCLE**

By GERTY T. CORI AND CARL F. CORI

**A COMPARISON OF GLUTATHIONE AND CYSTINE AS SUPPLE-
MENTING AGENTS IN DIETS DEFICIENT IN CYSTINE**

By C. J. STUCKY AND ERWIN BRAND

BROMOPHENYLMERCAPTURIC ACID SYNTHESIS IN THE DOG

By ABRAHAM WHITE AND HOWARD B. LEWIS

THE VALIDITY OF THE VISCOMETRIC AND WOHLGEMUTH METHODS FOR THE QUANTITATIVE DETERMINATION OF AMYLASE

By L. C. CHESLEY

(From the Zoological Laboratory, Duke University, Durham)

(Received for publication, March 14, 1931)

The sole means of measuring quantitatively the enzyme content of biological material consists in the determination of the catalytic activity of that preparation.

That the results of various workers might be more closely comparable, much work has been done in an effort to standardize the conditions of the experiments; optimal temperatures and ion concentrations have been investigated, and when determined, their maintenance has been incorporated in the standard method for enzyme measurements.

The kinetics of amylase action have been difficultly susceptible of analysis, for it is by no means certain that amylase is a single enzyme. Indeed there is evidence that it is a mixture of two or even three distinct enzymes. The starch used as substrate does not have a uniform composition, but is made up of several substances, chiefly α -amylose (amylocellulose, amylopectin) and β -amylose (amylose, amylopectin) as well as dextrans.

In view of the fact that these components vary, proportionately, in different commercial starches, and that β -amylose is more readily hydrolyzed than is the α -amylose, it seemed possible that the results obtained when different starches were used as substrates, all other conditions being kept constant, would not be similar.

Four lots of soluble starch were used in the experiments to be described. Two of these were soluble starch, c.p., Baker's analyzed, and two were Lintner's soluble starch.

The methods investigated were the much used Wohlgemuth method (5) and the newer viscometric method of Davison (2).

A. The Wohlgemuth Method

This method is based on the disappearance of the blue color given by starch and iodine as the starch is hydrolyzed. Progressive quantities of enzyme solution are placed in a series of test-tubes, and 5 ml. of ice-cold 1 per cent starch solution are added to each tube. All are then simultaneously placed in an incubator and allowed to digest for an arbitrary period of time, after which they are removed, and each tube filled nearly to the top with ice water. A drop of 0.1 N iodine is added to each tube. The lowest limit of activity lies in the last tube showing a nuance of blue.

If this tube contained 0.02 ml. of enzyme solution, then 0.02 ml. of that solution was capable of hydrolyzing 5 ml. of 1 per cent starch to dextrins, and by calculation, 1.0 ml. of the enzyme solution would hydrolyze 250 ml. of 1 per cent starch to the dextrin stage.

The units, in which the enzyme quantity is expressed, are the number of ml. of 1 per cent starch that 1.0 ml. of the enzyme solution would convert to dextrins. In the expression are included the conditions of the experiment; *i.e.*, time and temperature. In the example cited, the number of units is 250, and is expressed: $D_{\frac{\text{temperature}}{\text{time}}} = 250$.

In the present series of experiments, three starch samples were used. Due to the extreme rapidity with which the blue color disappeared in one of these, very dilute solutions of saliva were used.

For starch Samples 1 and 2 the amounts of enzyme solution used in a typical experiment were as follows: 0.15, 0.30, and 0.45 ml.—saliva diluted 1:40 with distilled water.

For starch Sample 3 the quantities were 0.01, 0.02, 0.04, and 0.06 ml. of the same enzyme solution. The three digestion experiments were conducted simultaneously, at the same temperature of 35°.

The pH was adjusted to 6.9 by means of 0.05 M phosphate buffer mixtures. The buffer solution was made up as described by Clark (1), 6 parts of 0.1 M Na_2HPO_4 being used with 4 parts of 0.1 M KH_2PO_4 . This 0.1 M phosphate mixture was added to an equal volume of 2 per cent starch, and the pH checked colorimetrically.

If a method be valid, the quantitative values of enzyme found in a biological material should be reproducible by other investigators, with, of course, different starches.

This experiment serves as an index to the reproducibility of such determinations. The results obtained in all three cases should be closely comparable as the *same enzyme solution* is used in each. However the different starches gave widely discrepant results, as is shown in Table I.

It will be noticed that different dilutions of the enzyme were used in different experiments, and that for starch Sample 3 values are not given at the lower dilutions. The widely discordant results obtained with the different starch samples could not be foreseen, and in the first experiments the enzyme quantity used, slight though it was, was great enough to digest starch Sample 3 beyond the end-point (and even beyond the achromatic point).

The variation in $D_{30'}^{35^\circ}$ between starch Samples 2 and 3 is seen to

TABLE I
Wohlgemuth Method Showing Variation in the Different Starch Samples
Values for $D_{30'}^{35^\circ}$.

Dilution	Starch Sample 1	Starch Sample 2	Starch Sample 3	Ratio
1:40	444	220	5000	2:1:23
1:40	667	267	5000+	2 8:1:20-
1:30	375	217	7500	1.7:1:34
1:20	416	226	5000+	1.8:1:26-
1:10	417	209	5000	2:1:24
1:3	750	375	1500+	2:1: <i>x</i>
1:5	312	178	1250+	1.7:1: <i>x</i>

be quite consistently in the neighborhood of 2400 per cent, and between Samples 1 and 2, consistently 100 per cent.

Wohlgemuth (5) originally specified that the quantity of enzyme solution in successive test-tubes should progress geometrically rather than arithmetically as in these experiments. Evans (3) has pointed out that the digestion factor would then progress geometrically and thus introduce a considerable error. In the present series the enzyme amount has been arithmetically increased, thereby eliminating this error.

B. The Viscometric Method

The viscosity of a solution depends upon the particle size of the dispersed phase. As the scission of the starch molecule into dextrans proceeds, the viscosity decreases. Following this decrease in viscosity, the digestion is measured. In this method a unit of

amylase is the amount of enzyme which will cause a reduction of 20 per cent in the viscosity in 60 minutes, or 10 per cent in 19.2 minutes, or 5 per cent in 8.4 minutes. Thus if 0.2 ml. of the enzyme solution should cause a reduction to 80 per cent of the original viscosity in 20 minutes, then the solution would contain $\frac{90}{20} \times 5 = 15$ units of amylase per ml. A starch solution of such concentration as to give a viscosity $2\frac{1}{2}$ times as great as that of water is most accurate, according to Davison. For most lots of Lintner's soluble starch, a 2 to 6 per cent solution gives the required viscosity.

The concentrations used in these experiments were as follows:

Sample 1.....	9.0 per cent (Baker's)
" 2.....	4 5 " " (Lintner's)
" 3.....	20 5 " " "
" 4.....	6.5 " " (Baker's)

Obviously, starch Sample 3 is unfitted for use as substrate in this method.

It was this starch sample which gave the anomalous results in the experiments with the Wohlgemuth method. The cause of this will be discussed later.

10 ml. of starch solution were placed in each of three Ostwald viscosimeters, and 1 ml. of 0.55 M NaCl added to each. 0.2 ml. of enzyme solution (dilute saliva) was added and well mixed with the starch. The viscosity was noted at 10 minute intervals.

Three starch samples were used simultaneously, the temperature was kept at 35°, and pH at 6.9 by 0.05 M phosphate buffers as previously described. Six viscosimeters were operated at once, three containing the different starch samples and active enzyme, and three serving as controls, one for each sample.

The results obtained are shown in Table II.

The variation between starch Samples 2 and 3 is seen to be about 1800 per cent, that between Samples 1 and 2 about 450 per cent, and between Samples 3 and 4 about 1800 per cent. This indicates that if very nearly the same concentration of different starches is required to give the viscosity of 2.5, the results will be comparable. However, if there is much of a discrepancy between concentrations, a considerable error results. When this factor is controlled, the viscometric method becomes reliable, and is probably the most convenient of any method in use, as the readings can be taken at any desired interval.

It seems reasonably certain that the discordant results obtained with the different starch samples are due to varying proportions of β -amylose in each. It will be noticed that the order of digestibility is exactly reversed in the two series of experiments; this rules out inhibitory substances present in different amounts.

The blue color given by starch and iodine is due only to the β -amylose.

During digestion β -amylose is precipitated out of solution in those starch samples in which it is found in any considerable quantity.

In starch Sample 3, the blue color very readily disappears, and

TABLE II

Comparison of Starch Samples as Substrates for Viscometric Determination
Amylase units per ml. of saliva.

Dilution	Starch Sample 1	Starch Sample 2	Starch Sample 3	Starch Sample 4	Ratio
1:1			1 1	18 7	1:17
1:1			1 2	20 0	1:16 7
1:1			1 0	20 0	1:20
1:8	16 0	38 0	1 7		9 4:21 1:1
1:8	21 5		0 7		30 7: x :1
1:8	17 5	37 5	0 75		23 3:50:1
1:4	25 7	90 0	6 12		4 0:14 5:1
1:4	26 7	120 0	6 5		4 1:18 4:1
1:6	24 5	140 0	7 0		3 5:20:1
Concentration, per cent	9 0	4 5	20 5	6 5	

in prolonged digestion no precipitate appears in the solution. This is indicative of a paucity of β -amylose in this starch.

α -Amylose is the constituent giving the greatest viscosity. The fact that a concentration of 20.5 per cent of starch Sample 3 is required to give the viscosity of 2.5 indicates that the starch must be, in the main, made up of dextrins. This view is strengthened by the observation that there is relatively a very slight decrease in viscosity under the action of saliva.

From these considerations it is evident that the reason for the great "digestibility," or the early disappearance of blue color, in starch Sample 3 is that it has relatively very little of the β -amylose, and this is quickly hydrolyzed.

It is suggested that the separated α - and β -amyloses would constitute better substrates than does soluble starch.

Sherman and Baker (4) have compared the separated components with Lintner's starch and concluded that Lintner's starch is a valid substrate. However, they use starch prepared in their own laboratory which would doubtless be more nearly uniform than are commercial starches from different sources.

It is clearly recognized that in the case of starch Sample 3 we are dealing with an extreme case; but it is also clear that in most of the current methods this would not be noticed, unless the extremely large enzyme values were compared with values obtained for the same material with a different starch as substrate.

The variation in the four starch samples investigated indicates that results obtained by workers using these methods are at best difficultly comparable.

Comparisons of copper reduction and polariscopic methods, as well as the development of methods employing the separated constituents of starch are now in progress in this laboratory.

SUMMARY

1. Four starch samples have been compared as to digestibility as measured by the Wohlgemuth and viscometric methods.

2. The same amount of enzyme was added to each starch solution. However the amount of enzyme as reckoned from its activity varied from 100 to 2400 per cent among the different substrates when measured by the Wohlgemuth method, and from 350 to 2100 per cent as measured by the viscometric method.

3. It is suggested that the varying results given by the different starch samples are due to their differential digestibility, and this in turn to the varying proportions of α - and β -amyloses and the dextrins.

I wish to acknowledge my indebtedness to Professor F. G. Hall, under whose direction this work was conducted.

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STUDIES ON CRYSTALLINE UREASE

III. THE TOXICITY OF CRYSTALLINE UREASE

By HENRY TAUBER AND ISRAEL S. KLEINER

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INTRODUCTION

Reports in the literature about the toxicity and action of urease in the animal organism are not in agreement. According to Carnot and associates (1) urease rapidly destroys the urea contained in the blood if injected into it and causes intoxication and death of the animal resulting from the ammonemia produced. Lövgren (2) states categorically, without publishing experiments or other evidence, that urease has a toxic effect upon the animal organism, whether subcutaneously or intravenously injected. Lublin (3) finds that urease or urea injected separately into rabbits or mice causes no marked disturbance, but simultaneous injection of the two substances is followed by death. He could not find any evidence of the conversion of urea into ammonium carbonate in the blood and attributes the cause of death to emboli and not to ammonia poisoning. This seems to be substantiated by his finding that the red blood cells of rabbits are agglutinated by urease *in vitro*, and by his assertion that the action of urease is too slow at so low a temperature as 37° to cause rapid death by ammonia poisoning. Sunzeri (4) states that urease exerts its expected action when injected into the circulatory system of the dog, only if the ureters are ligated. The apparent lack of action in dogs with unligated ureters is due to the elimination of the enzyme in the urine. Rigoni (5) injected soy bean extracts into rabbits intravenously and found that certain amounts of urease solutions provoke an intense ureolytic action. The animals die within a few hours with symptoms of ammonia intoxication.

Urea disappears in the blood almost completely, while large amounts of ammonia nitrogen accumulate in the blood and organs. The blood and liver of such animals show an intense ureolytic power. He also states that, if an animal survives one injection, it will tolerate much larger doses quite easily and offers the hypothesis that the liver has an inactivating action upon urease (6). According to Taketa (7), urease acts as a diuretic when injected intraperitoneally into rabbits; if urea is first injected intravenously, followed by intraperitoneal injection of urease, there is a marked pharmacological action characterized by weakness, convulsions, and acidosis.

Since the work of the authors mentioned above was done with impure, crude urease extracts and the results are contradictory, it seemed of interest to repeat these experiments using crystalline urease which is relatively pure and highly active. It may be standardized and, if toxic, the lethal dose determined. It was also our hope to throw some light on the mechanism of the toxic action of urease.

We prepared crystalline urease from jack bean meal according to Sumner's method (8). For some of the experiments the same lot of meal was used as was employed in a recently published study by one of us (9). For the others we used an Arlco jack bean meal (No. 300411) which was unusually rich in urease, containing 180 units per gm. of dry meal compared with meals previously employed, which contained 135 units or less.

Determination of Activity of Jack Bean Meal and Urease Solutions

For the determination of the activity of jack bean meal and urease solutions we modified slightly the method of Sumner (8). 0.2 to 0.3 gm. of powdered meal was weighed out into a 150 cc. Erlenmeyer flask and 20 cc. of distilled water were added at 20°. After the meal was mixed well with the water, the flask was immersed in a water bath at 20°. Then 20 cc. of "urea-phosphate" solution, which had been brought to 20°, were added. The urea-phosphate solution contained 3 per cent urea, 5.4 per cent Na_2HPO_4 , and 4.2 per cent KH_2PO_4 and was of pH 7. After mixing the contents of the flask it was left for 5 minutes in the water bath. After 5 minutes 20 cc. of N hydrochloric acid were added to stop the enzyme action. The contents of the flask were

mixed and 1 cc. transferred to a 100 cc. volumetric flask. To this 2 cc. of gum ghatti, prepared according to Folin and Svedberg (10), and about 75 cc. of distilled water were added and direct Nesslerization was employed. As in the original method, about 1 mg. of ammonia nitrogen should be formed by the urease for each cc. of urea-phosphate. The result is expressed as the number of units of urease per gm. of meal. The unit as defined by Sumner is the amount of urease which will produce 1 mg. of ammonia nitrogen from urea in 5 minutes at 20°.

Preparation of Urease Solutions for Injection

Fresh urease solutions were prepared every day, because the aqueous solutions do not keep well. The crystals from 100 gm. of jack bean meal were dissolved in 25 cc. of distilled water at 20° and centrifuged free from insoluble matter. This solution was diluted further with distilled water or with 0.9 per cent sodium chloride so as to contain 1 or 5 units of urease per cc., respectively. Since urease is not very soluble in 0.9 per cent sodium chloride, we used distilled water for the solution containing 5 units per cc. The pH of these solutions was 7.2 as determined by the colorimetric method. We have noticed that success in crystallizing urease depends largely on the use of redistilled acetone and also upon efficient refrigeration. Crystallization sometimes takes more than 24 hours and we found that the size of the crystals increases if the filtrate of the extract is left in the refrigerator for several days.

Results

In Table I will be found a summary of all the experiments on mice. After non-lethal doses the animals show little more than somnolence. With larger doses, this is often followed (sometimes preceded) by restlessness and labored breathing, and later by violent convulsions. Death usually results after a period of coma. The minimal lethal dose is about 0.09 unit per gm. of body weight. Apparently a tolerance is acquired if a second, or even a third injection is given at 24 hour intervals after a non-lethal dose. In two instances these second and third injections exceeded greatly the minimal lethal dose. This seems to substantiate the findings of Rigoni, who used soy bean extracts.

The symptoms produced in mice and rabbits are quite similar to

TABLE I
Experiments on Mice. Subcutaneous Injection of Urease

Experiment No.	Weight of mouse gm.	Concentration of solution per cc.	Amount injected per gm of body weight	Time before death		First injection	Remarks
				hrs.	Recovered		
1	29	1	0.086				
1	29	1	0.172			after 24 hrs.	Sleepiness during the first 2 hrs.
1	29	5	0.260			" 48	"
2	31	1	0.064				"
2	31	1	0.128			after 24 hrs.	"
2	31	5	1.600			" 48	"
3	24	1	0.042				"
3	24	5	0.070			after 24 hrs.	"
3	24	5	0.520	19		" 48	"
4	24	1	0.126	12			Labored breathing, sleepiness
5	25	1	0.120	6			Violent convulsions followed by coma and death
6	22	5	0.080	4		"	"
7	26	5	0.086	9		"	"
8	25	5	0.120	20		"	"
9	25	5	0.240	16		"	"
10	28	5	0.350	22		"	"

those produced by ammonium salts, although perhaps not identical with them. A few control experiments were performed in which ammonium carbonate was injected into mice and rabbits. These results are shown in Tables II and IV.

Larger amounts of urease were used in the experiments on rabbits. From 10 to 40 units were injected intravenously into rabbits weighing about 2 kilos each. Blood was taken from the ear vein before injection and 5 and 15 minutes afterwards. Table III shows the results of analysis of these samples. Folin and Svedberg's (10) method for urea was used, while ammonia was determined by precipitating the proteins immediately by the Folin-Wu procedure and Nesslerizing an aliquot part of the filtrate directly.

TABLE II

Experiments on Mice. Subcutaneous Injection of Ammonium Carbonate

Experi- ment No.	Weight of mouse	Amount injected	Strength of solution	Remarks
	<i>gm.</i>	<i>cc.</i>	<i>per cent</i>	
11	27.5	2.00	10	Labored breathing, sleepiness, violent convulsions, followed by coma and death
12	25.0	2.00	10	Labored breathing, sleepiness, violent convulsions, followed by coma and death
13	31.0	0.50	10	Sleepiness, one hind leg paralyzed, convulsions, followed by recovery
14	28.0	0.25	10	Sleepiness, convulsions, recovery

In 5 minutes a very large part of the blood urea has been transformed to ammonia and by 15 minutes all has been so changed. 1 hour after the injection, urease is still demonstrable in the blood but none is to be found in the urine. Apparently symptoms and death were caused by ammonia poisoning. Corroboration of this is given by several tests on rabbits, in which ammonium carbonate was injected, and which are summed up in Table IV. By comparison of the results of Tables III and IV it appears that although the concentration of NH_3 in the blood of animals injected with $(\text{NH}_4)_2\text{CO}_3$ is quite high for a few minutes it rapidly falls and after 15 minutes it is considerably lower than that in the urease-injected animals. Apparently death from ammonia poisoning

TABLE III
Experiments on Rabbits. Intravenous Injection of Urease

Experiment No.	Weight	Concentration of solution per cc.	Amount injected	Time before death	Blood						Urea in blood after 1 hr.	Urea in urine	Remarks	
					Urea N			Ammonia N						
					Before injection	After 5 min.	After 15 min.	Before injection	After 5 min.	After 15 min.				Before death
			cc.		mg.	mg.	mg.	mg.	mg.	mg.				
1	2302	1	10	18 hrs.	13.0	4.0	None	0.10	8	13		Large	None	
2	2300	1	10	22 "	12.0	3.5	"	0.2	9.4	12		amount	"	Hind legs paralyzed
3	1962	5	8	3 hrs., 15 min.	11.5	3.0	"	0.15	17	18.5	9	"	"	
4	1894	5	8	3 hrs., 5 min.	12.5	2.5	"	0.15	17	17	12	"	"	

TABLE IV
Experiments on Rabbits. Injection of Ammonium Carbonate

Experiment No.	Weight gm.	Concentration of solution per cent	Amount injected cc.	Manner of injection	Time before death	Ammonium N in blood				Remarks
						Before injection mg.	After 5 min. mg.	After 15 min. mg.	Shortly before death mg.	
52700	10	10	6	Intravenous	50 min.					Convulsions after 5 min. of injection, labored breathing, sleepiness, coma
62750	10	10	6	"	80 "					
72230	10	10	0.0022 per kg. body weight	"	95 "	0.245.0	2.6	2.0		
82010	10	10	0.0022 per kg. body weight	"	3 hrs., 32 min.	0.122.0	4.0	3.5		"
92450	10	10	10	Subcutaneous*	Recover- ed	0.112.0	0.2			Moderately severe symptoms
	10	10	20	"	3 hrs., 30 min.	0.117.0	3.2	3.0		Second injection 2 days after first
102335	10	10	20	"	5 hrs., 25 min.	0.218.5	3.8	3.5		Symptoms similar to those in Experiment 5

* Simultaneously in several places.

may occur with much lower blood ammonia than was present after urease injection.

DISCUSSION

Our results point directly to ammonia intoxication as the cause of death after injection of urease parenterally. The failure of some of the earlier workers to demonstrate this was due, no doubt, to the use of urease of low potency. Even the employment of amounts of urease much greater than the minimal lethal dose did not give any different symptoms, so that we may assume that urease has no other pharmacological action than that of producing the toxic ammonia. Our experiments with crystalline urease thus substantiate those of Carnot, Gerard, and Moissonnier and of Lövgren with crude material. Lublin's statement that both urease and urea must be injected together is not corroborated. There is sufficient urea present in the blood and tissues to yield a fatal dose of ammonia. Furthermore, in another paper (11) it has been demonstrated that, although he is correct in his assertion that urease has agglutinating properties *in vitro*, this cannot be the cause of death because the agglutination is completely inhibited in the presence of serum.

Rigoni's experiment of producing a tolerance to urease by repeated injections seems to be substantiated by the few experiments which we have made. However, the mechanism of this phenomenon is not quite clear and we hope to continue along this line at some future time.

SUMMARY

Crystalline urease is toxic when injected subcutaneously into mice or intravenously into rabbits. The minimal lethal dose for mice is about 0.09 unit per gm. of body weight. The blood urea is quickly and completely transformed into ammonia, which apparently is the toxic agent, since the symptoms closely resemble those exhibited in ammonia poisoning.

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THE CALCIUM-PHOSPHORUS RATIO OF THE TIBIÆ OF GROWING CHICKS*

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Until quite recently chickens were raised under conditions more or less similar to the conditions under which wild birds live. The chicks, which were hatched in the spring, were grown on free range during the summer months. Thus they were provided with an abundance of green plants and unlimited sunshine which possessed the maximum antirachitic activity. Under those conditions problems concerning chick nutrition and the prevalence of malnutrition among chicks were relatively rare as compared with the present day situation. Furthermore, the chicks grown under those conditions were permitted to grow at a much slower rate than chicks which are now raised under the more intensive conditions employed by the modern poultry industry.

At the present time it is essential that chicks shall grow at a rapid rate while receiving a diminished amount of range and sunshine and that the females shall mature early and subsequently produce a continuous large yield of eggs over a 12 or 14 months period. Furthermore, the females are frequently expected to produce eggs of high hatchability which will yield a good number of vigorous chicks of high viability. These demands create a variety of types of malnutrition and present an intricate problem of poultry nutrition.

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In considering the preparation of rations suited for the promotion of good growth and bone development in young chicks, attention obviously must be given to the protein, mineral, vitamin, and carbohydrate components of the ration. It has been recognized for some time that the rate of growth may be materially influenced by the amount of protein in the ration. However, increasing the protein content of the ration and thereby increasing the rate of growth, particularly if the chicks are closely confined, frequently causes rickets and slipped tendon (hock trouble) (Fig. 1). To counteract rickets (leg weakness) it has been the practice of poultrymen and feed manufacturers to incorporate cod liver oil



FIG 1 Showing chick with hock trouble

in the ration as a source of vitamin A and vitamin D and to add various amounts of calcium-rich materials to insure an abundance of calcium.

It was pointed out in a previous paper (1) that the addition of various calcium-rich materials may be followed by unsatisfactory results if the amount of calcium is excessive or if the ratio of calcium to phosphorus is too high. Also it was pointed out that the best growth and development were obtained when the calcium-phosphorus ratio was less than 2.00:1.00. In the present paper attention is directed to the possible effect of various factors such as the ash content of the ration, the ratio of calcium to phosphorus in the ration, the rate of growth of chicks, and the ash

content of the tibiae on the calcium-phosphorus ratio of the tibiae of growing chicks.

For the purpose of this study, Rhode Island Red chicks were raised from hatching to 9 weeks of age in battery brooders (Fig. 2)

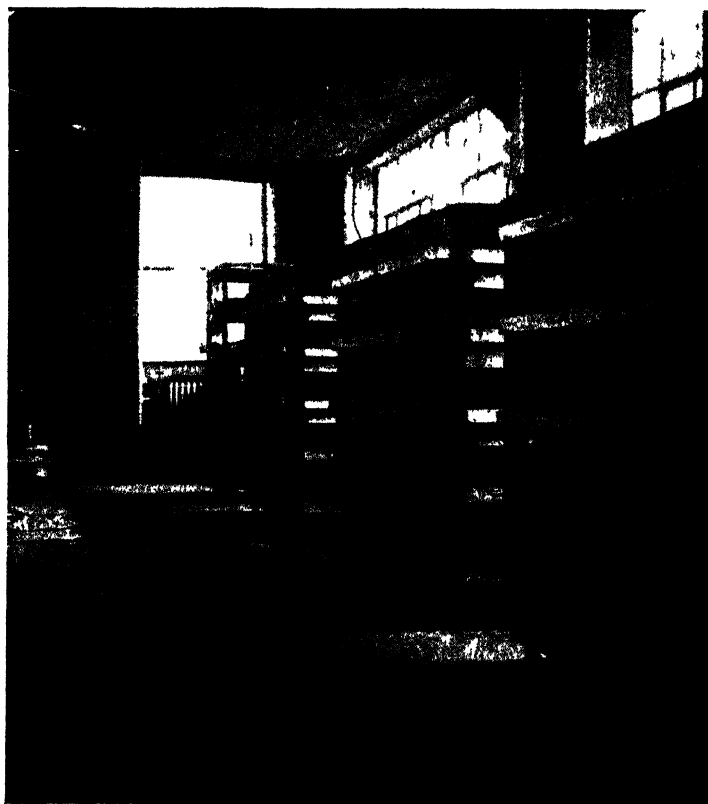


FIG 2 Showing battery brooders in which the chicks used in this study were raised under conditions similar to those used in commercial plants producing broilers.

under conditions similar to those used in commercial plants producing broilers.

Chicks in pens of forty each were fed all mash rations which insured all birds receiving the desired proportion of food con-

stituents. The experimental rations were so constituted that they contained varying amounts of protein, vitamins, and minerals, thus providing an opportunity for noting the effect of such variations on the calcium-phosphorus ratio of the tibiæ. The chicks were fed and watered daily and the ration and chicks were weighed at weekly intervals. At the end of the 3rd, 6th, and 9th weeks, five birds, typical of the pen, were removed from each pen for study of bone development. The left tibiæ were dissected, freed of tissue, dried at 100°, measured (length and diameter), weighed, ashed, and the calcium and phosphorus content of the ash was determined. From these data, the ratio of calcium to phosphorus for each of the twenty-nine pens of chicks under discussion was determined for the chicks at 3, 6, and 9 weeks of age. These data are assembled in Table I, the calcium-phosphorus ratios for the chicks at 3 weeks being arranged in an ascending order. It will be noted that the calcium-phosphorus ratio for most pens varies for the three determinations. In some instances, the ratio increases with the age of the chicks. In some instances the calcium-phosphorus ratio decreases as the age increases and in some instances the ratio increases and subsequently decreases. The variation in the calcium-phosphorus ratio is least at 9 weeks of age, the minimum being 1.88:1.00 and the maximum being 2.18:1.00. It is interesting to note that the average of each period is the same, being 2.02:1.00 at 3 weeks of age, 2.03:1.00 at 6 weeks of age, and 2.03:1.00 at 9 weeks of age.

Since it is a common commercial practice to add various sources of calcium, such as edible steamed bone, oyster shells, limestone, and similar materials to chick rations for the purpose of increasing the calcium content, it is of interest to note the effect of variation in the ash content of rations upon the calcium-phosphorus ratio of the growing chicks. In Table II data have been assembled for rations which contained from 6.64 to 13.47 per cent of ash. On inspecting Table II, it will be noted that the calcium-phosphorus ratio of 2.01:1.00 for the ration containing 6.64 per cent ash is the same as the calcium-phosphorus ratio of 2.00:1.00 for the ration containing 11.50 per cent of ash; that the calcium-phosphorus ratio of 2.04:1.00 for the ration containing 8.00 per cent of ash is the same as that of 2.05:1.00 for the ration containing 13.40 per cent of ash; that the calcium-phosphorus ratio of

1.99:1.00 for the ration containing 8.00 per cent of ash is the same as that of 1.98:1.00 for the ration containing 11.05 per cent of ash; and that the calcium-phosphorus ratio of 2.08:1.00 for the ration containing 8.30 per cent of ash is the same as that of

TABLE I
Ca:P Ratio of Tibiæ from Chicks at Different Ages

Pen No.	Ca:P ratio at 3 wks.	Ca:P ratio at 6 wks.	Ca:P ratio at 9 wks.
1	1.87:1.00	2.07:1.00	1.88:1.00
VI	1.89:1.00	1.94:1.00	1.93:1.00
V	1.90:1.00	1.92:1.00	1.92:1.00
2	1.95:1.00	2.02:1.00	2.05:1.00
F	1.96:1.00	1.99:1.00	2.06:1.00
G	1.96:1.00	2.03:1.00	2.05:1.00
M	1.96:1.00	2.03:1.00	2.08:1.00
L	1.97:1.00	2.04:1.00	2.00:1.00
D	1.98:1.00	2.04:1.00	2.01:1.00
O	1.98:1.00	1.98:1.00	2.04:1.00
11	1.99:1.00	1.95:1.00	1.98:1.00
B	2.01:1.00	2.01:1.00	1.99:1.00
H	2.01:1.00	2.03:1.00	2.07:1.00
I	2.01:1.00	2.03:1.00	2.09:1.00
J	2.01:1.00	1.99:1.00	2.03:1.00
K	2.01:1.00	2.01:1.00	2.07:1.00
6	2.01:1.00	2.02:1.00	2.01:1.00
7	2.01:1.00	2.13:1.00	1.90:1.00
A	2.03:1.00	2.01:1.00	2.12:1.00
N	2.03:1.00	1.98:1.00	1.99:1.00
10	2.04:1.00	2.00:1.00	2.04:1.00
C	2.04:1.00	2.04:1.00	2.07:1.00
E	2.05:1.00	1.98:1.00	2.06:1.00
8	2.09:1.00	2.17:1.00	2.06:1.00
14	2.09:1.00	2.09:1.00	2.18:1.00
9	2.12:1.00	2.09:1.00	2.14:1.00
15	2.13:1.00	2.09:1.00	2.05:1.00
13	2.21:1.00	2.12:1.00	2.14:1.00
12	2.32:1.00	1.86:1.00	2.11:1.00

2.09:1.00 for the ration containing 11.90 per cent of ash. In brief, a careful consideration of Table II reveals that there is no constant increase in the calcium-phosphorus ratio of the tibiae of 9 weeks old chicks corresponding to the increase in the per cent of ash in the rations

The possible influence of ash content of the ration on the calcium-phosphorus ratio of the tibiæ having been considered, it is of interest to consider the relation of the calcium-phosphorus ratio of the rations to the calcium-phosphorus ratio of tibiæ of chicks which were grown on the rations. Accordingly, the calcium-phosphorus ratios of the various rations have been arranged in ascending order (Table III). The minimum calcium-phosphorus ratio was 1.10:1.00 for the ration fed to the chicks in Pen 7 and the maximum calcium-phosphorus ratio was 5.16:1.00 for the ration fed to those in Pen 9. Thus the ratio of calcium-

TABLE II
Relation of Ash Content of Ration to Ca P Ratio of Chick Tibiæ

Pen No	Ash in ration	Ca P ratio of tibiæ	Pen No	Ash in ration	Ca P ratio of tibiæ
	<i>per cent</i>			<i>per cent</i>	
6	6 64	2 01:1 00	A	10 50	2 12:1 00
7	6 66	1 90:1 00	B	10 60	1 99:1 00
VI	7 00	1 93:1 00	C	10 60	2 07:1 00
N	8 00	1 99:1 00	E	10 60	2 06:1 00
O	8 00	2 04:1 00	12	11 01	2 11:1 00
M	8 30	2 08:1 00	9	11 03	2 14:1 00
V	8 45	1 92:1 00	11	11 05	1 98:1 00
K	8 50	2 07:1 00	10	11 06	2 04:1 00
H	8 60	2 07:1 00	8	11 08	2 06:1 00
2	9 80	2 05:1 00	13	11 10	2 14:1 00
1	9 89	1 88:1 00	L	11 50	2 00:1 00
D	9 90	2 01:1 00	I	11 90	2 09:1 00
J	9 90	2 03:1 00	15	13 40	2 05:1 00
F	10 00	2 06:1 00	14	13 47	2 18:1 00
G	10 40	2 05:1 00			

phosphorus was increased 5-fold. However, this excessive variation of the calcium-phosphorus ratio did not produce any corresponding influence on the calcium-phosphorus ratio of the tibiæ.

The various rations under consideration produced widely different results as regards growth, feathering, pigmentation, and physical appearance of the birds (Fig. 3). In view of the radical difference in body increment of chicks in various pens, it is of interest to consider the calcium-phosphorus ratio of tibiæ taken from 9 weeks old chicks which have grown at materially different rates. Table IV reports these data in which the final weight or

the weight of the 9 weeks old chicks of the different pens has been arranged in ascending order. The chicks which grew at the slowest rate weighed $\frac{3}{4}$ of a pound at 9 weeks of age. The chicks which grew most rapidly weighed $2\frac{1}{2}$ pounds at 9 weeks of age.

TABLE III
Relation of Ca:P Ratio in Ration to Ca:P Ratio of Chick Tibiæ

Pen No.	Ca P ratio of ration	Ca in ration	P in ration	Ca:P ratio of tibiæ
		<i>per cent</i>	<i>per cent</i>	
7	1.10:1.00	0.95	0.86	1.90:1.00
6	1.12:1.00	0.99	0.88	2.01:1.00
VI	1.23:1.00	1.24	1.01	1.93:1.00
N	1.31:1.00	1.60	1.22	1.99:1.00
11	1.34:1.00	3.80	2.83	1.98:1.00
10	1.35:1.00	3.80	2.82	2.04:1.00
O	1.35:1.00	1.66	1.23	2.04:1.00
M	1.35:1.00	1.73	1.28	2.08:1.00
J	1.46:1.00	2.19	1.50	2.03:1.00
G	1.48:1.00	2.27	1.53	2.05:1.00
K	1.49:1.00	1.91	1.28	2.07:1.00
F	1.49:1.00	2.14	1.44	2.06:1.00
D	1.51:1.00	2.07	1.37	2.01:1.00
L	1.51:1.00	2.24	1.48	2.00:1.00
2	1.51:1.00	2.18	1.44	2.05:1.00
E	1.52:1.00	2.27	1.49	2.06:1.00
H	1.53:1.00	1.91	1.25	2.07:1.00
B	1.55:1.00	2.36	1.52	1.99:1.00
1	1.62:1.00	2.29	1.41	1.88:1.00
C	1.64:1.00	2.26	1.38	2.07:1.00
I	1.76:1.00	2.50	1.42	2.09:1.00
A	1.87:1.00	2.51	1.34	2.12:1.00
V	2.11:1.00	2.23	1.05	1.92:1.00
12	4.65:1.00	3.86	0.83	2.11:1.00
15	4.88:1.00	4.10	0.84	2.05:1.00
13	5.02:1.00	3.97	0.79	2.14:1.00
14	5.06:1.00	4.15	0.82	2.18:1.00
8	5.08:1.00	3.86	0.76	2.06:1.00
9	5.16:1.00	3.87	0.75	2.14:1.00

While a number of factors were involved, the two factors which undoubtedly greatly influenced the difference in the rate of growth were the variation of protein and vitamin content of the ration. However, it is interesting to note that the chicks which weighed

under 1 pound at 9 weeks of age and which were in a most unsatisfactory physical condition produced tibiæ which had approximately the same calcium-phosphorus ratio as the chicks which weighed more than twice as much at the same age. From these observations it is apparent that the rate of growth exerts very little influence on the calcium-phosphorus ratio of the tibiæ of growing chicks.

Inasmuch as the chicks of different pens varied materially in size when 9 weeks of age, a question naturally arose as to the extent of development of the bones removed from the chicks of the various pens. Since in some laboratories the ash content of tibiæ is relied upon for determining the presence or absence of



FIG. 3. Showing the widely different results as regards growth, feathering, pigmentation, and physical appearance of chicks raised on different rations.

rickets in growing chicks, it is of more than passing interest to compare the tibia ash content with the calcium-phosphorus ratio of the tibiæ. Accordingly, the ash content of the tibiæ of dried, extracted bone has been arranged in Table V in an increasing sequence. The least amount of ash present in the tibiæ of chicks was 34.99 per cent for Pen 13. The greatest amount of ash was 49.12 per cent for Pen 6. Strange to relate, the calcium-phosphorus ratio for the tibiæ with the minimum amount of ash exceeded materially the calcium-phosphorus ratio of the tibiæ with the maximum ash content. In Table V, the column headed "Extent of calcification" contains a summary of the results of the histological examina-

tion of the right tibiae corresponding to the left tibiae used for the ash, calcium, and phosphorus determinations.

For the sake of brevity, an attempt has been made to summarize in one or two words, the results of the histological examination of the several right tibiae of the chicks of each test group. Unfortunately, this procedure may perhaps in some instances convey a different impression than if a more detailed description of the internal structure were given; *i.e.*, "irregular" which is used to describe tibiae of chicks from Pens L, E, J, N, B, has been sub-

TABLE IV
Relation of Body Weight of Chicks to Ca:P Ratio of Chick Tibiae

Pen No	Weight of chicks	Condition of chicks	Ca P ratio of tibiae	Pen No.	Weight of chicks	Condition of chicks	Ca P ratio of tibiae
	lbs.				lbs.		
7	0 74	Deplorable	1 90:1.00	B	1.52	Fair	1.99:1.00
1	0 84	"	1.88:1.00	N	1.56	"	1.99:1.00
11	0 96	"	1.98:1.00	14	1 58	"	2.18:1.00
E	1.11	Very poor	2 06:1.00	D	1.64	Good	2 01:1.00
13	1.16	" "	2.14:1.00	F	1.67	"	2.06:1.00
J	1.32	Inferior	2 03:1.00	K	1.71	"	2.07:1.00
12	1 37	"	2 11:1.00	I	1.79	"	2.09:1.00
L	1.38	"	2 00:1.00	M	1 91	Very good	2 08:1.00
G	1 38	"	2 05:1.00	2	1 91	" "	2.05:1.00
9	1.40	"	2 14:1.00	O	2.02	" "	2.04:1.00
8	1.41	"	2 06:1.00	10	2.05	" "	2.04:1.00
A	1 47	Fair	2 12:1.00	6	2.05	" "	2.01:1.00
C	1.48	"	2 07:1.00	V	2.25	Excellent	1.92:1.00
H	1.50	"	2 07:1.00	VI	2 36	"	1.93:1.00
15	1 50	"	2 05:1.00				

stituted for "Very wide epiphyseal cartilage, irregular calcification and a tendency towards thickening of the shaft." Similarly the terms used to describe the extent of calcification of tibiae of chicks from Pens K, G, H, A, V, 6, and VI should be amplified if the histological condition of the tibiae was to receive major consideration in this discussion. Instead, attention is being primarily directed towards a consideration of the mineral content of the tibiae and reference to the histological condition of the tibiae is being made for the purpose of raising a question as to the reliability of the ash content of the tibiae as the sole criterion for determining the presence

or absence of rickets. In some circles, it is a practice to consider that 42.00 per cent of ash in the tibia is a definite line of demarcation; *i.e.*, all chicks having a tibia ash content of under 42.00 per cent should be considered as rachitic and those chicks having a

TABLE V
Relation of Ash Content of Tibiæ to Their Ca:P Ratio

Pen No.	Ash in tibiæ	Extent of calcification	Ca:P ratio
	<i>per cent</i>		
13	34.99	Very poor	2.14:1.00
12	35.80	Poor	2.11:1.00
7	36.35	"	1.90:1.00
1	37.28	"	1.88:1.00
9	37.75	"	2.14:1.00
11	38.47	Very poor	1.98:1.00
L	40.42	Irregular	2.00:1.00
E	40.63	"	2.06:1.00
15	40.68	Poor	2.05:1.00
J	43.50	Irregular	2.03:1.00
N	43.89	"	1.99:1.00
B	44.42	"	1.92:1.00
I	44.54	Normal	2.09:1.00
D	44.95	"	2.01:1.00
K	45.12	Slightly abnormal	2.07:1.00
C	45.73	Normal	2.07:1.00
G	45.83	Slightly abnormal	2.05:1.00
M	46.12	Normal	2.08:1.00
2	46.21	"	2.05:1.00
H	46.23	2 normal, 2 rachitic	2.07:1.00
A	46.43	Slightly abnormal	2.12:1.00
O	46.57	Normal	2.04:1.00
V	47.07	4 normal	1.92:1.00
8	47.14	Normal	2.06:1.00
F	47.38	"	2.06:1.00
6	48.45	4 normal	2.01:1.00
10	48.67	Normal	2.04:1.00
14	48.82	4 normal	2.18:1.00
VI	49.12	4 "	1.93:1.00

tibia ash content of 42.00 per cent or more should be considered as having normal bone structure. In this laboratory, it has not been possible to place sole reliance on tibia ash content to distinguish between chicks having rachitic or normal bone development

and, furthermore, it has not been possible unreservedly to use a tibia ash content of 42.00 per cent as a sharp line of demarcation between rachitic and normal bone development. Data on this point are being constantly collected and it is expected that a forthcoming paper will discuss this matter in detail.

A careful comparison of the calcium-phosphorus ratio obtained for the tibiae of chicks from the twenty-nine pens under consideration with the ash content of those from corresponding pens shows that there is no correlation of the calcium-phosphorus ratio of the tibiae corresponding to the decided variation in the per cent of ash in the tibiae.

DISCUSSION

Inasmuch as there appears to be no correlation between the calcium-phosphorus ratio of the tibiae of 9 weeks old chicks with the per cent of ash in the ration, with the calcium-phosphorus ratio of the ration, with the body weight of the chicks, or with the ash present in the tibiae, it would seem that calcium and phosphorus are deposited in a fairly constant ratio regardless of the completeness or incompleteness of bone development.

Furthermore, it would seem to follow from the above that the present day practice of adding quite appreciable amounts of ground limestone or other calcium-rich materials to rations for growing chicks may be followed by unsatisfactory results and that it is preferable to select and proportion the ingredients of chick rations so that the desired calcium level will be maintained and the ratio will not greatly exceed 2 parts of calcium¹ to 1 part of phosphorus.

This conclusion having been reached, the next step obviously was to test the soundness of the conclusion. Accordingly, a series of chick mashes was prepared which contained less calcium than is ordinarily present in commercial mashes and in which the calcium-phosphorus ratio did not exceed 2 parts of calcium to 1 part of phosphorus. Several pens of chicks have already been

¹ The literature contains few data concerning the calcium-phosphorus ratio of the tibiae of growing chicks. Buckner, Martin, and Insko (2) report a variation of the calcium-phosphorus ratio of chick tibiae from 2.02:1.00 to 2.06:1.00 for six pens of 10 weeks old chicks. Wilgus (3) found that the calcium-phosphorus ratio of the tibiae of 8 weeks old chicks varied from 1.84:1.00 to 2.07:1.00 for chicks grown on a variety of rations.

grown on this series of mash. The results which have thus far been obtained for the calcium-phosphorus ratio of the tibiæ are far more consistent than those reported in the preceding pages for the twenty-nine pens under discussion. For the twenty-nine pens the calcium-phosphorus ratio of the tibiæ varied from a minimum of 1.88:1.00 to a maximum of 2.18:1.00 or a difference of 0.30:1.00. With the new series of mash, the variation from the minimum to the maximum calcium-phosphorus ratio has not exceeded one-third this amount. Several pens of chicks are now being grown on the new series of mash and shortly considerable data should be available bearing on this point.

SUMMARY

Twenty-nine pens of Rhode Island Red chicks have been grown in commercial battery brooders from hatching to 9 weeks of age. They were fed the all mash type of ration. The different mash varied in protein, mineral, and vitamin content. At weekly intervals the chicks and mash were weighed. When the chicks were 3, 6, and 9 weeks old, five representative chicks were selected from each pen. They were killed and the tibiæ were removed. The left tibiæ were dried, measured (length and diameter), weighed, ashed, and the ash analyzed for calcium and phosphorus.

The calcium-phosphorus ratio of the tibiæ of the 9 weeks old chicks was compared with the ash content of the rations, the calcium-phosphorus ratio of the ration, the rate of growth of the chick, and the ash content of the tibiæ.

The results of this study indicate that there is no correlation between the calcium-phosphorus ratio of the tibiæ of 9 weeks old chicks with the per cent of ash in the ration, with the calcium-phosphorus ratio of the ration, with the body weight of the chicks, or with the ash present in the tibiæ. Thus it would seem that calcium and phosphorus are deposited in a fairly constant ratio regardless of the completeness or incompleteness of bone development.

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DETERMINATION OF ALCOHOLIC INTOXICATION DURING LIFE BY SPINAL FLUID ANALYSIS

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In a paper (1) published from our laboratories, Gettler and Tiber described a method for the quantitative estimation of the alcohol content of the brain, lungs, and liver. In another paper (2) the same authors showed that the quantity of alcohol in the brain determines the degree to which an individual is affected by the alcohol—an index of intoxication. It is evident, however, that this method is applicable only to cadavers. In seeking a method for proving intoxication in living individuals, it occurred to us that perhaps the alcohol content of the blood or spinal fluid would be of value. The problem confronting us was to analyze the brain, blood, and spinal fluid of cadavers, and the blood and spinal fluid of living alcohol drinkers, and then determine whether there is any constant relation between the alcohol content of the brain and that of the blood or of the spinal fluid. It is upon this work that we wish to report.

The method used in this investigation is the same as that outlined by Gettler and Tiber (1) for the brain and other tissues. Certain details of the method had to be modified because the amounts used for analysis were much smaller, the blood is a coagulating fluid, and the acidity control may have a different value. The changes introduced were as follows:

10 cc. of blood or spinal fluid were used for the analysis.

Paraffin was used to prevent frothing; it served the purpose better than white mineral oil.

Steam distillation was made use of. The flask containing the blood or spinal fluid, however, was not heated. Steam alone was used for heating and distilling. In this way, in the case of blood,

an ungainly coagulum adhering to the flask was avoided. The steam gradually coagulated the blood, while in motion, disintegrating the coagulum into small particles.

Amount of Distillate Required to Recover All of the Alcohol Present

10 cc. portions of blood and of spinal fluid from cases in which the brain showed a high alcohol content were distilled with steam. The distillate was collected in successive 10 cc. portions, until 100 cc. were obtained. Each 10 cc. portion of distillate was oxidized and then tested with reduced fuchsin. The results are tabulated in Table I. It was found that all the alcohol was recovered in the first 70 cc. of distillate.

TABLE I

Amount of Distillate Necessary for Complete Recovery of Alcohol from Blood or Spinal Fluid

Case No.	Alcohol content of brain	Alcohol found in successive 10 cc. portions of distillate*						
		0-10	10-20	20-30	30-40	40-50	50-60	60-70
	<i>per cent</i>							
1	0.38	+++	++	Trace	None	None	None	None
2	0.36	+++	++	+	Slight trace	"	"	"
3	0.4	+++	++	Trace	" "	"	"	"
4	0.38	+++	++	++	+	Trace	Trace	"
5	0.6	++++	++++	++	+	+	"	Trace
6	0.55	++++	++++	++	+	+	"	"
7	0.4	+++	++	Trace	None	None	None	None

* All determinations on successive 10 cc. portions of distillate from 70 to 100 gave negative results.

Determination of the Best Conditions for Oxidation

A series of 100 cc. portions of water, each containing 50 mg. of alcohol, was used. Varying amounts of dichromate and sulfuric acid were added. On distilling, it was possible to obtain 80 cc. of distillate, no trace of sulfur trioxide passing into the distillate. The acetic acid in the distillate, produced by oxidation of the alcohol, was titrated. The results are given in Table II.

The optimum amount of oxidizing mixture for 100 cc. of alcohol solution (containing in the neighborhood of 50 mg. of alcohol) is 7.5 gm. of potassium dichromate and 15 cc. of concentrated sulfuric acid.

Method in Detail for the Quantitative Estimation of Ethyl Alcohol in Blood and Spinal Fluid

10 cc. of the fluid and 1 gm. of paraffin are placed in a 100 cc. distillation flask which is then set up for steam distillation. The flask containing the blood or spinal fluid is not heated. The steam alone is used for the distillation. 100 cc. of distillate are collected. To these 100 cc. of distillate, placed in a 200 cc. round bottom flask, are added 7.5 gm. of potassium dichromate and 15 cc. of concentrated sulfuric acid. The flask is connected to a long, well cooled condenser by means of a Hopkins distilling head. The contents are well mixed. A medium sized ($1\frac{1}{2}$ inch) Bunsen flame is applied, so that the distillation is not too rapid. The distillation should be at a slow but constant rate. It should take about 60 minutes to

TABLE II

Best Conditions for Oxidation of Solutions Containing 50 Mg. of Added C_2H_5OH

$K_2Cr_2O_7$ in oxidizing mixture	H_2SO_4 in oxidizing mixture	0.05 N acetic acid in distillate	C_2H_5OH recovered	
gm.	cc.	cc.	mg.	per cent
5.5	11	18.95	43.6	87.2
6.5	13	19.00	43.7	87.4
7.5	15	19.80	45.5	91.0
8.5	17	19.25	44.2	88.4
10.0	20	18.90	43.4	86.8

collect the required 80 cc. of distillate. When exactly 80 cc. have been collected, the distillate is well mixed and aliquot portions are titrated with 0.05 N sodium hydroxide for acetic acid produced. A small part of this distillate should be tested for the sulfate ion, in order to be sure that no sulfur trioxide passed into the distillate. If the above procedure is followed, there should be absolutely no danger of sulfur trioxide distilling. From the amount of acetic acid found by titration, the amount of ethyl alcohol is calculated.

Acidity Produced by Oxidation of the Distillate from Non-Alcoholic Spinal Fluid

It was essential to determine whether the spinal fluid of individuals who have had absolutely no alcohol will give a distillate

which will yield acidity upon oxidation. Spinal fluid samples from ten cases were analyzed according to the method outlined above. The patients having been in the hospital under observation, we were positive that they had not had any alcohol for at least a fortnight prior to the removal of the spinal fluid. The results of the analyses are given in Table III.

The figures in Table III indicate that non-alcoholic spinal fluid yields certain volatile substances to the distillate, which, when oxidized in accordance with our method, yield on the average for every 10 cc. of spinal fluid an acidity of 0.78 cc. 0.02 N. This is equiva-

TABLE III

Acidity Produced by Oxidation of Spinal Fluid Distillate from Non-Alcoholic Individuals

10 cc. of spinal fluid were used for each analysis.

Case No.	Source of specimen	0.02 N acidity produced	Alcohol equivalent
		cc.	per cent
1	From autopsy	0.92	0.0083
2	" hospital patient	1.00	0.0090
3	" " "	0.76	0.0068
4	" " "	0.70	0.0063
5	" " "	0.78	0.0070
6	" " "	0.72	0.0065
7	" autopsy	0.70	0.0071
8	" hospital patient	0.78	0.0070
9	" " "	0.70	0.0063
10	" autopsy	0.74	0.0067
Average.....		0.78	0.0071

lent to 0.0071 per cent of ethyl alcohol, or 0.71 mg. of ethyl alcohol in every 10 cc. of spinal fluid. Whether this is actually ethyl alcohol one cannot definitely state. The amount present is too small to be identified. We simply claim that it is acidity produced by the oxidation of some normal volatile substances.

Acidity Produced by Oxidation of the Blood Distillate from Non-Alcoholic Individuals

In order to determine whether distillates from blood, upon oxidation by our method, yield any acidity, the following experiment was conducted.

Specimens of blood from fourteen cases were analyzed according to our method. The fourteen patients were under observation in the hospital and had partaken of no alcohol for at least a fortnight prior to the drawing of the blood. The results are shown in Table IV.

The experiments given in Table IV indicate that non-alcoholic bloods yield certain volatile substances to the distillate, which, when oxidized by our method, yield on an average for every 10 cc.

TABLE IV

Acidity Produced by Oxidation of Blood Distillates from Non-Alcoholic Individuals

10 cc. of blood were used for each analysis.

Case No.	Source of specimen	0.02 N acidity produced	Alcohol equivalent
		cc.	per cent
1	From hospital patient	1.10	0.0099
2	" " "	1.60	0.0144
3	" autopsy	1.45	0.0130
4	" hospital patient	0.80	0.0072
5	" " "	2.40	0.0216
6	" " "	1.60	0.0144
7	" " "	1.80	0.0162
8	" " "	1.24	0.0111
9	" " "	1.58	0.0142
10	" " "	0.92	0.0083
11	" " "	0.80	0.0072
12	" " "	1.65	0.0148
13	" autopsy	0.45	0.0041
14	" " "	1.30	0.0117
Average.....		1.33	0.0120

of blood an acidity of 1.33 cc. 0.02 N, equivalent to 0.012 per cent of ethyl alcohol, or 1.2 mg. of ethyl alcohol in 10 cc. of blood. We do not assert at present that this is ethyl alcohol; it is some volatile material found in normal blood which, upon oxidation, yields titratable acidity.

Table III indicates that from 10 cc. of non-alcoholic spinal fluid an average acidity of 0.78 cc. 0.02 N is produced. Table IV shows that from 10 cc. of non-alcoholic blood an average of 1.33 0.02 N

acidity is produced. These values must be subtracted from all titration figures when the amount of alcohol present in blood or spinal fluid is calculated. In blood and spinal fluid of alcoholics, we titrated the acidity produced with 0.05 N alkali; therefore, the values to be subtracted are 0.31 cc. 0.05 N for spinal fluid and 0.53 cc. 0.05 N for blood. Although there is a difference of 0.22 cc. 0.05 N between these two values, yet in contrast with the much larger values obtained from alcoholic blood and spinal fluid (15 to 20 cc. 0.05 N), the above difference of 0.22 cc. 0.05 N is negligible. For this reason we averaged the values obtained in spinal fluid (0.31 cc. 0.05 N) and in blood (0.53 cc. 0.05 N) and

TABLE V
Recovery of Alcohol from Spinal Fluid and Blood

50 mg. of alcohol were added to each specimen.

Sample No.	Specimen	0.05 N acidity produced	0.05 N acidity due to alcohol added	Alcohol recovered	
		cc.	cc.	mg.	per cent
1	Blood	19.67	19.25	44.27	88.54
2	"	19.57	19.15	44.04	88.08
3	"	18.89	18.47	42.48	84.96
4	"	18.84	18.42	42.37	84.74
5	Spinal fluid	18.77	18.35	42.20	84.40
6	" "	18.34	17.92	41.22	82.44
7	" "	18.40	17.98	41.35	82.70
8	" "	18.93	18.51	42.57	85.14
Average recovery.....				42.56	85.12

obtained the value 0.42 cc. 0.05 N. This value we subtracted from all alcohol determinations in blood and spinal fluid.

Amount of Alcohol Recoverable from Spinal Fluid and Blood

The object of this experiment was to determine how much of added alcohol could be recovered from blood and spinal fluid. 10 cc. portions of non-alcoholic spinal fluid and blood were used. 50 mg. of absolute alcohol were added to each portion. The procedure of analysis was as outlined above. The results are tabulated in Table V.

As a result of this series of experiments, we find that the optimum

average recovery of alcohol from blood and spinal fluid to which known quantities of alcohol were added is 85 per cent.

Calculation—From the titration figures, the amount of alcohol in spinal fluid is calculated as follows:

$$\text{Per cent alcohol} = (\text{cc. } 0.05 \text{ N alkali used} - 0.42) \times \frac{100}{85} \times 0.0023 \times 10$$

in which 0.42 is the blank control; $\frac{100}{85}$, the factor based on 85 per cent recovery; 0.0023, the gm. of alcohol equivalent to 1 cc. (0.05 N); and the multiple 10 is used to get the amount in 100 cc. from the 10 cc. used.

Alcohol Content of Brain, Blood, and Spinal Fluid

The alcohol content of the brain, blood, and spinal fluid in a series of cases was determined by the method previously outlined. The results are given in Table VI.

Gettler and Tiber (2) have shown that the alcohol content of the brain can be used to prove intoxication. We, therefore, used the figures obtained from the brain as the basis of our blood:brain ratio and spinal fluid:brain ratio. A comparative study of the figures in Table VI shows the following.

1. The alcoholic content of the spinal fluid is always higher than that of the brain.

2. The smaller the alcohol content of the brain, the larger is the spinal fluid:brain ratio. The larger the amount of alcohol in the brain, the nearer do the spinal fluid values approach the values in the brain, the spinal fluid:brain ratio approaching a value of 1.1.

3. The spinal fluid:brain ratio in intoxicated individuals is below 1.4 (it ranges between 1.4 and 1.1).

4. The spinal fluid:brain ratio in individuals who have partaken of some alcohol, but in whom the amount accumulated in the brain is too small to cause intoxication, is above 1.5 (it may go as high as 3).

5. The alcohol content of the blood varies greatly. In some of our cases it was higher than that of the brain and in other cases it was lower.

6. The blood:brain ratios range anywhere from 0.66 to 4.55. There is no regularity as in the case of the spinal fluid:brain ratio.

This variation evidently depends upon the stage of absorption at which death intervenes.

From the facts determined above, we claim that the spinal fluid, but not the blood, can be used for estimating the amount of

TABLE VI
Comparative Study of the Alcohol Content of Brain, Blood, and Spinal Fluid

Case No.	Brain alcohol	Blood alcohol	Blood ratio Brain	Spinal fluid alcohol	Spinal fluid ratio Brain	Remarks
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>		
1	0.012	0.021	1.85	0.032	2.66	Coronary sclerosis; found dead
2	0.018	0.078	4.33	0.039	2.16	Heart case; found dead
3	0.033	0.023	0.69	0.094	2.85	" " " "
4	0.117	0.103	0.88	0.177	1.51	Jumped from roof
5	0.118			0.178	1.51	Had alcohol, but not intoxicated; lived 3 hrs. in hospital
6	0.220*	0.324	1.47	0.265	1.20	Seen intoxicated; found dead $\frac{1}{2}$ hr. later
7	0.224*	0.224	1.00	0.266	1.18	Seen intoxicated; found dead 1 hr. later
8	0.256*	0.161	0.63	0.322	1.25	Seen intoxicated; dead 2 hrs. later
9	0.283*	0.292	1.03	0.364	1.28	Seen intoxicated; found dead 4 hrs. later
10	0.305*	0.394	1.29	0.393	1.28	Intoxicated; fell from roof
11	0.307*	0.380	1.23			Seen intoxicated; died in street
12	0.318*	0.345	1.08	0.399	1.25	Intoxicated; then found dead
13	0.369*	0.404	1.09	0.462	1.25	At friend's home; intoxicated; collapsed and died
14	0.427*	0.475	1.20	0.542	1.27	Seen intoxicated; found dead
15	0.489*	0.461	0.94	0.545	1.11	Intoxicated at friend's home; died shortly after
16	0.580*	0.460	0.80	0.645	1.11	Seen in coma; then died

* Intoxicated.

alcohol in the brain of a living individual. The alcohol content of the brain and that of the spinal fluid run quite parallel. Since the alcohol content of the brain is an index of intoxication, the spinal fluid can be used to prove intoxication in living individuals.

The spinal fluid:brain ratios being used, we suggest that the following procedure be used for estimating the amount of alcohol in the brain of a living individual.

For alcohol content of spinal fluid	(0.03-0.10 per cent)	divide by 2.5
" " " " " "	(0.15-0.20 " ")	" " 1.5
" " " " " "	(0.25-0.50 " " " "	" " 1.2
	(0.50-0.70	1.1

TABLE VII

Comparative Study of the Alcoholic Content of Spinal Fluid and Blood of Hospital Patients

Case No.	Spinal fluid alcohol	Blood alcohol	Blood Spinal fluid ratio	Remarks
	<i>per cent</i>	<i>per cent</i>		
1	0.070	0.055	0.78	Not intoxicated
2	0.087	0.052	0.60	" "
3	0.205	0.695	3.39	Boisterous but equilibrium seemed stable
4	0.272	0.203	0.74	Intoxicated
5	0.295	0.163	0.55	"
6	0.314	0.027	0.08(?)	"
7	0.354	0.353	1.00	"
8	0.431	1.032	2.39	"
9	0.438	0.250	0.57	"
10	0.443	0.405	0.91	"
11	0.447	0.227	0.51	"
12	0.538	0.416	0.77	Coma
13	0.657	0.351	0.53	"
14	0.696	0.251	0.36	"
15	0.741	0.511	0.69	"

Alcohol Content of Blood and Spinal Fluid of Hospital Patients

The alcohol content of the blood and spinal fluid in a series of sixteen hospital cases was determined, the methods previously outlined being used. The results are given in Table VII. The values obtained indicate the following.

1. All cases having 0.272 per cent or more alcohol in the spinal fluid were intoxicated. This corroborates the findings of Gettler and Tiber (2) that all individuals with an alcohol content of over 0.25 per cent in the brain are intoxicated. All those cases in which

the alcohol content of the brain was below 0.2 per cent were not intoxicated. Gettler and Tiber (2) found that some cases in which the alcohol content of the brain was between 0.2 per cent and 0.25 per cent showed slight disturbance in equilibrium, while others did not. Through an oversight, however, this finding was not pointed out in their original paper and we, therefore, call attention to it at this time.

2. The alcoholic blood values are very irregular. The blood:spinal fluid ratio varies from 0.08 to 3.39.

3. The alcohol content of the spinal fluid (but not the blood) can be used as an index of intoxication. Individuals having 0.272 per cent of alcohol or more in the spinal fluid can be safely regarded as intoxicated.

SUMMARY

1. A quantitative method for ethyl alcohol determination in blood and spinal fluid is described. The alcohol is isolated by steam distillation. It is quantitatively estimated by oxidizing it to acetic acid and titrating the acid thus produced.

2. By the use of this method, the alcohol content of blood and spinal fluid of alcoholic hospital patients and also of alcoholics who had been autopsied was determined. In the latter group the brain also was analyzed for alcohol.

3. The alcohol content of the spinal fluid is always somewhat higher than that of the brain. There is, however, a definite and regular relation between the alcohol content of the brain and spinal fluid.

4. The alcohol content of the blood varies greatly when compared with that of the brain. It may be higher or it may be lower than the alcohol content of the brain.

5. From the values obtained by analysis, the blood:brain and spinal fluid:brain alcoholic ratios were calculated.

6. The smaller the alcohol content of the brain, the larger is the spinal fluid:brain ratio, reaching a value of 2.85. The larger the amount of alcohol in the brain, the nearer do the spinal fluid values approach the values in the brain, the spinal fluid:brain ratio approaching a value of 1.1.

7. The spinal fluid:brain ratio in intoxicated individuals is below 1.4 (it ranges between 1.4 and 1.1).

8. The spinal fluid:brain ratio in individuals who have partaken of some alcohol, but where the amount accumulated in the brain is small and, therefore, not enough to cause intoxication, is above 1.4 and may run as high as 3.

9. The blood:brain ratios vary anywhere between 0.66 and 4.55. There is no regularity in the blood:brain ratios as there is in the spinal fluid:brain ratios.

10. All cases having 0.265 per cent or more alcohol in the spinal fluid were intoxicated. This corroborates the findings of Gettler and Tiber that individuals with an alcohol content in the brain of over 0.25 per cent are intoxicated.

11. A method is suggested for estimating the alcohol content of the brain of a living individual from the alcohol content determined in the spinal fluid. Dividing the alcohol content of the spinal fluid by the spinal fluid:brain ratio, we obtain a close estimate of the alcohol content of the brain.

12. The alcohol content (0.265 per cent and higher) of the spinal fluid of living persons is an index of intoxication.

We wish to acknowledge our appreciation of the courtesy of Dr. Alexander Lambert, Director of the Fourth Medical Division of Bellevue Hospital, in permitting us to use the clinical material from his service.

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THE FORMATION OF SEMIQUINONES AS INTERMEDIARY REDUCTION PRODUCTS FROM PYOCYANINE AND SOME OTHER DYESTUFFS

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INTRODUCTION

In a recent study of the blue pigment of *Bacillus pyocyaneus* (1), it was observed that this dyestuff, in alkaline solution, can be reversibly reduced in the same way as any organic dye of the quinoid type, whereas in acid solution the reduction goes on in two successive steps, each characterized by an individual color and an individual potential. This behavior recalls the two step reduction observed by Willstätter and Piccard (2) for iminoquinone. But there is, according to what is known about the latter, a difference in the nature of the intermediary compound in the two cases. For iminoquinone the intermediary compound is considered by Willstätter as a molecular compound of the fully reduced and the fully oxidized states. This intermediary compound belongs to the quinhydrones, or more generally speaking, to what Willstätter and Piccard designated as meriquinones.¹ In contrast to this, in the case of pyocyanine the reduction goes on, according to the previous communication, in such a way that the intermediary compound is a true intermediary state of oxidation, with the same molecular size, without the formation of a molecular compound. In the case of pyocyanine, there is a simple reversible two step oxidation, each step involving the loss of 1 hydrogen atom. Such a case is, to my knowledge, encountered in the literature in one instance: Cannan (5) showed that hermidine, a vegetable pigment extracted from *Mercurialis perennis*, of un-

¹ However, cf. Hantzsch's (3) and Weitz and Fischer's (4) point of view as referred to in a later section of this paper.

known chemical constitution, is reduced in two successive steps, each involving the loss of 1 hydrogen atom and characterized by a particular color. Findings of this kind should not be considered as mere curiosities, but rather as something typical for certain dyestuffs. One cannot refrain from the speculation that this two step reduction must have a bearing both on the biological side of oxidation-reduction, and on the pure chemistry of dyestuffs at the same time. The first thing to do was to find some more dyestuffs of the same behavior. After several futile attempts two more dyestuffs were discovered which showed the same property. There can be little doubt that many others can be found² after the general principle of the matter is illuminated in a certain respect in this paper. One of these two is α -oxyphenazine, synthesized in 1928 by Wrede and Strack (6). It is worth mentioning that Wrede and Strack were unable to obtain the hydroxy compound by diazotizing Kehrman's amino compound but had to prepare it in a quite different way. I am indebted to Professor Wrede of Greifswald for a specimen of his α -oxyphenazine. This dye is, according to Wrede and Strack, closely related to pyocyanine, which is nothing but a methylated α -oxyphenazine. The very surprising discovery of a phenazine derivative among the products of metabolism of a living organism seems to be confirmed beyond doubt by Wrede and Strack's synthesis of pyocyanine by methylizing α -oxyphenazine, in 1929. There is only one detail in which the formulation of pyocyanine of these authors seems to me inadequate, namely the bimolecular structure. The following experiments will show that there is no room for a bimolecular formula in pyocyanine in aqueous solution. The freezing point determinations by Wrede and Strack, which are in favor of the bimolecular formula, hold for organic solvents, but they prove nothing for aqueous solutions if the reasoning to be presented in this paper is acceptable. When $C_{13}H_{10}N_2O$ is taken for pyocyanine (the half of Wrede's formula) and $C_{12}H_8N_2O$ for α -oxyphenazine, according to Wrede's analysis in 1928, it becomes most likely that pyocyanine is nothing but monomethyloxyphenazine. The fact described by Wrede, that in strongly alkaline solution pyocyanine is

² Several examples of this kind may be found in Hantzsch's paper (3). It is remarkable that these examples also belong to the derivatives of phenazine.

partially converted to α -oxyphenazine, recalls the demethylation of methylene blue in alkaline solutions. No splitting of any polymerized form of pyocyanine need be assumed.

The other of the dyes is rosinduline and was studied in a previous communication (7) for the oxidation-reduction potential between pH 4 and 12. There was, then, no particular interest in extending this investigation to a more acid range. Now, however, this extension was performed, and in very acid solutions this dye showed the same behavior as pyocyanine.

It is a common feature of all these three dyes, pyocyanine, oxyphenazine, and rosinduline, that the splitting of the reduction process into two steps occurs only in acid solution. The two successive potential levels are more and more separated from each other as the pH becomes smaller. For pyocyanine, the separation begins to become manifest at pH about 5.5; for oxyphenazine at pH 3.8; for rosinduline not before pH 2.0. At pH values sufficiently smaller than the limiting values just given, the separation of the two potential levels is very distinct and no appreciable overlapping takes place. Each of the two steps of the titration curve has the appearance of a reversible oxidation system with the electron number 1, as it is in the case of ferricyanide-ferrocyanide, or ferripyrophosphate-ferropyrrophosphate (8), and in contrast to the behavior of an ordinary organic dye in which what we called the electron number is always 2.

As to the common feature in the chemical constitution of these three dyes, at the present time the hint may suffice that all of them can be conceived of as derivatives of monoimidoquinone, $\text{NH}=\text{C}_6\text{H}_4=\text{O}$, in part of the orthoquinoid, in part of the paraquinoid type.

The difference between 1 molecule of each of the two successive steps will be proved to be only 1 electron (*i.e.* hydrogen atom) without any change in molecular size. The intermediary form will in this case be designated as a semiquinone. This is in contrast to that intermediary form which is designated as a quinhydrone, or more generally, as a meriquinone, and which is a molecular compound of the totally reduced form and the oxidized, holoquinoid form. We shall always in this paper use the words semiquinone and meriquinone with this distinction. The decision as to whether the intermediary form is a semiquinone or

a meriquinone can be attained by a mathematical analysis of the titration curve in the following way.

Theory of the Titration Curves for the Case of Semiquinone and of Meriquinone

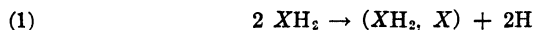
The case in which the two step oxidation is due to the formation of a semiquinone can be expressed by Scheme A.

Scheme A



The case of the formation of a meriquinone can be expressed by Scheme B, where the meriquinone is supposed to have the simplest constitution, namely that of a quinhydrone (hydroquinone and holoquinone in the ratio 1:1).

Scheme B



Depending on whether the reaction goes on according to Scheme A or B, the shape of the titration curve will be different. In all considerations we shall start with the completely reduced dye and titrate it with an oxidant. There will be two potential levels in either case. In general, the formula expressing the potential as a function of the amounts of the dye in its various possible forms of existence—the oxidized, intermediary, and reduced states—and of pH is extremely complicated. One may see from the paper on meriquinones by Clark and Cohen (9) how unhandy the formulæ become for the case of a meriquinone. They are not so very much simpler for the case of a semiquinone. It will not be necessary to develop these formulæ here, for the following reason. All difficulties entirely vanish if the two levels of the potential are distinctly separated; that is to say, if the oxidation of the intermediary form to the holoquinone does not begin, practically speaking, before the oxidation of the completely reduced form to the intermediary form has been finished. In all of our cases, this condition is fulfilled at a sufficiently low pH. We can restrict ourselves, therefore, to this case, and we may, furthermore, restrict ourselves to the consideration of the first half of the titration.

The second step, then, is a kind of repetition of the first. It will be a perfect repetition in the case of a semiquinone, except, of course, for the level of the potential. It will be what may be called an antisymmetric repetition in the case of a meriquinone, where each step of the titration curve is asymmetrically shaped around its particular mid-point. At any rate it is sufficient to analyze only one step of the oxidation in detail.

First Case. Formation of Semiquinone—Let the process go on according to Scheme A. Then the potential, during the first step of titration is simply this:

$$E = E_0 + \frac{RT}{F} \ln \frac{x}{a-x} = E_0 + 0.06 \log \frac{x}{a-x} \quad (1)$$

where x is the amount of oxidant used for the titration, counted in equivalents (1 equivalent being that amount which accepts 1 gm. of hydrogen), and a the initial amount of the dye, in mols.

Second Case. Formation of a Meriquinone—Let the process go on according to Scheme B. Then the chemical reaction going on reversibly at the electrode, during the first step of oxidation, will be:



and the potential will vary during the titration at constant pH according to the formula

$$E = E_0^* + \frac{RT}{2F} \ln \frac{[(\text{XH}_2, \text{X})]}{[\text{XH}_2]^2}$$

If in the beginning XH_2 amounts to a mols, after addition of x equivalents of the oxidant it will be $a - x$; and (XH_2, X) then will be $\frac{1}{2} x$. Hence

$$E = E_0^* + 0.03 \log \frac{\frac{1}{2} [x]}{[a-x]^2} = E_0^{**} + 0.03 \log \frac{[x]}{[a-x]^2}$$

or

$$E = E_0^{***} + 0.03 \log \frac{[x]}{[a-x]} - 0.03 \log [a-x] \quad (2)$$

This formula shows that the potential depends not only upon the ratio of concentrations of the completely reduced form and the

meriquinone, but also upon absolute concentrations. The general shape of this curve will be somewhat complicated, but in general the slope of such a curve will lie somewhat between an ordinary titration curve for the electron number 1, and such for the electron number 2. In Fig. 6 one curve has been calculated for the electron number 1, the other for 2. The meriquinone curve would lie between those. Its curvature would fit, in its left-hand part more to the curve $n = 2$, in its right-hand part more to the curve $n = 1$. So the curve would be somewhat asymmetrically arranged about its middle ordinate. The experimental curve, however, fits entirely to the curve $n = 1$.

Furthermore the meriquinone curve would depend on absolute values not only on ratios of concentrations. The easiest way of formulating this is as follows: When in formula (2) the concentration $[a - x]$ is replaced by $\frac{(a - x)}{v}$, where the parenthesis means absolute amount in mols, and v volume, then

$$E = E_0^{****} + 0.03 \log \frac{(x)}{(a - x)} - 0.03 \log (a - x)$$

Keeping all variables constant except for v , one obtains:

$$E = \text{constant} + 0.03 \log v$$

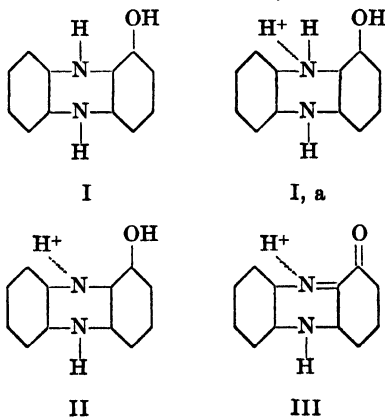
Let a given amount of the dye, amounting to a mols, be dissolved, for one experiment, in a definite volume of a buffer, and in another volume for another experiment. The dye is completely reduced and an oxidative titration performed for these two solutions. The two curves are plotted and compared, point after point. Then the potentials, for each particular value of x , in the two titration curves should differ by 30 millivolts when the volume of the one solution is 10 times that of the other. For practical purposes it was thought to be safer to compare one solution only with its 3-fold dilution, because the dye is so difficultly soluble that too high a dilution might have been disadvantageous for a rapid establishment of the potential. The difference of the potential should have amounted to $0.03 \times \log 3 = 0.0134$ volts. The experiment presented later on (Fig. 6), however, manifests no difference in the potentials at all in the two solutions.

According to Willstätter and Piccard (2), there is, furthermore, a possibility that a meriquinone is formed from its constituents in

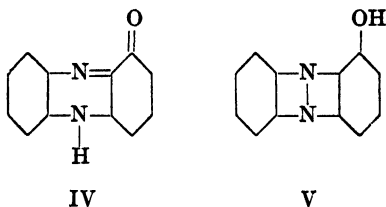
a ratio different from 1:1. On the supposition that this ratio was 1:2 or more, the displacement of the mid-point potential by variation of the volume would be still greater than in the case considered above. On the other hand, if this ratio were something like 2:3, the deviation of the whole curve from the one calculated for the ordinary case with the electron number of 1 would be still more conspicuous than in the simpler case. Thus, any kind of meriquinone formation can be excluded. This, of course, holds only for aqueous solution and need not be true for non-aqueous solutions or for the crystalline state.

Chemical Interpretation of the Intermediary Compound

Since the intermediary compound has been recognized as a semiquinone, the attempt has to be made to formulate such a compound. It must be a molecule of the character of a free radical, containing an odd number of electrons, like Gomberg's (10) triphenylmethyl, or Wieland's (11) di-aryl-nitrogen (see Lewis (12)). The following formulæ may serve as models for the case of oxyphenazine. Formula I is the completely reduced form: Formula I, a is the same as it exists in very acid solutions. It differs from Formula I in the same way as NH_4^+ from NH_3 . Formula II is the semiquinone, which exists only in acid solution and contains what we may call bivalent nitrogen (comparable to Wieland's bivalent nitrogen). Formula III is the completely oxidized form of the holoquinoid type, in acid solution. In less acid or alkaline solution the hydrogen ion is released from the nitrogen, and Formula IV arises.



Semiquinone Formation



In very alkaline solution a color change takes place indicating a further ionization of the oxidized form. This is made evident by applying Formula V which is tautomeric with Formula IV and contains a phenolic hydroxyl group. It is noteworthy that this tautomerism is not possible in the case of the methylated compound, pyocyanine. In accordance herewith, pyocyanine, in its oxidized form, shows no color change from a neutral to an extremely alkaline solution. (Something quite different and with no relation to this consideration is the fact that pyocyanine in very alkaline solution gradually undergoes an irreversible alteration attended by a change in color, as already described by Wrede and Strack.)

It is important to add that the radical-like compound, Formula II, is not a labile or metastable molecule but is in true equilibrium with the other forms, and that it cannot release the attached hydrogen ion without losing its possibility of permanent existence.

It seems likely that on reinvestigation of those compounds now considered as meriquinones, by applying the methods proposed, some of these compounds will turn out to be semiquinones instead of meriquinones. At any event, this method may be used for further investigations on many of those intermediary oxidation-reduction products, as those compiled for instance in Henrich's book (13), and more recently discussed by Weitz (14), and linked with the names of Gomberg, von Baeyer, Pfeiffer, Kauffmann, Willstätter, Hantzsch, Kehrman, Wieland, and many others. Especially, it should be pointed out that Weitz considers, quite generally, the quinhydrone not as molecular compounds but as radical-like single molecules, and that already Hantzsch (3) in 1916, strongly advocated a monomolecular formula for the quinhydrone. It may also be recalled that the potentiometric study by Conant, Small, and Taylor (15) on halochromic salts of triphenylmethyl belongs in this field.

It seems unlikely that the formation of a semiquinone as an intermediary product of reduction is restricted to the isolated cases presented in this paper. One may dare to express, as a working hypothesis for further investigations, the following idea. Quinoid substances may be able generally to form semiquinones. The conditions of their existence will be determined for aqueous solutions by a wedge-shaped field in a coordinate system, with pH and potentials as coordinates, in the same manner as the wedge designated as "green" in the figure in the paper on pyocyanine (1) or in Fig. 7 of this paper, or the field marked "violet" in Fig. 11 of this paper. The difficulty of generally proving this hypothesis may consist in the fact that the position of this wedge will often be in a part of the coordinate system which is not, or only difficultly, accessible for the experiment and where measurements of pH and oxidation-reduction potentials are uncertain. The wedge-shaped area may lie in a pH range corresponding to extremely concentrated strong acids, or in a potential range of overvoltage with respect to hydrogen or to oxygen. It seems likely that sometimes the area of existence of a semiquinone may lie outside the range of experimentally attainable conditions for aqueous solutions but inside the realizable conditions for non-aqueous solutions.

Description and Discussion of the Titration Curves

For the experiments with pyocyanine refer to the previous paper (1).

The methods for the two other dyes were the same as before. In all cases the dye was dissolved in the buffer solution. Both are only slightly soluble. With oxyphenazine the dye was dissolved in the gently heated buffer solution and filtered. With rosinduline the dye, in the form of the sodium salt, was dissolved in a drop of pure water, in which it is easily soluble unless pH is very low (< 3); then the acid (HCl or lactate buffer respectively) was added to the volume desired for the titration experiment. After the excess of dye had been precipitated in the constant temperature room at 30° , the solution was filtered and used for the titration. The solubility, at strongly acid reaction, is so low, that 20 cc. of the solution after reduction consumed altogether 0.70 cc. of 0.0005 M solution of quinone to complete reoxidation. Yet the potentials were fairly reproducible at different electrodes, at least in the well

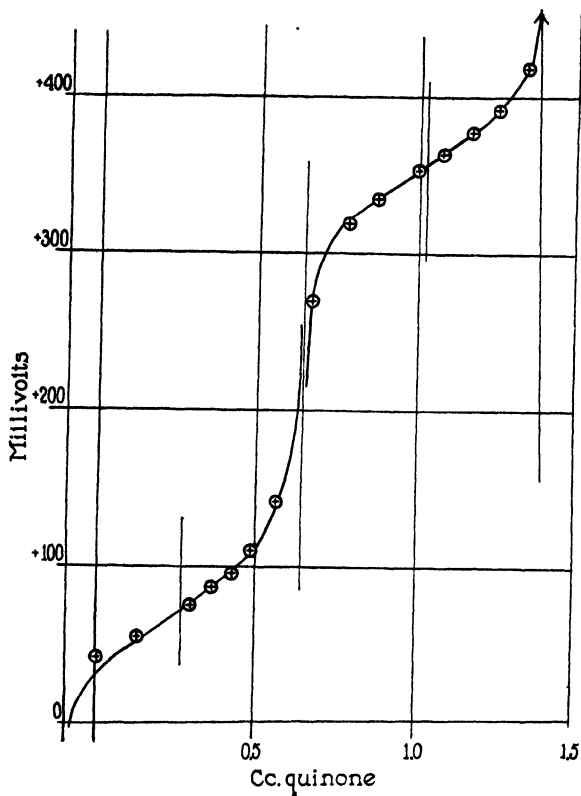


FIG. 1. α -Oxyphenazine, at pH 0.08 (approximately 1 *N* HCl), is completely reduced³ and titrated at 30° with a quinone solution (likewise in 1 *N* HCl). The abscissa indicates cc. of the quinone solution added; the ordinate is the potential, in millivolts. All potentials in this paper are referred to the normal hydrogen electrode. The drawn out curve is, for each of the two steps in the potential level, the one calculated for a reversible oxidation with the electron number 1. Concerning the extrapolation of this curve to the negative side of the abscissa and the deviation of the first (or the two first) observed values from the theoretical curve see the text. The separation of the two halves is very distinct.

³ During the state of complete reduction, in the atmosphere of pure hydrogen, the pH measurement was performed with a platinized electrode. All pH values in this paper are directly measured in this way; never was the pH value taken as it might have been approximately calculated from the composition of the buffer solution used. No correction, even in very acid solutions, was applied for the liquid junction potential between the buffer and the agar bridge saturated with KCl.

poised parts of the curves, though not quite with the same accuracy as was the case in the experiments with this dye at higher pH, as described in the previous paper, or with oxyphenazine in all pH ranges.

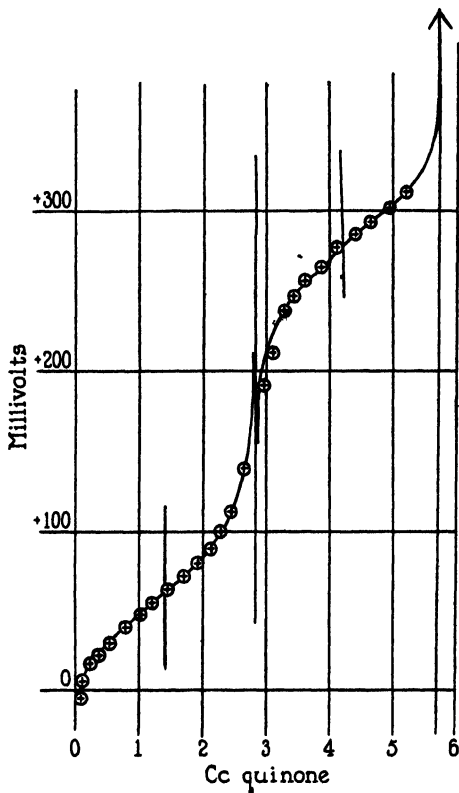


FIG. 2. α -Oxyphenazine dissolved in a solution of pH 1.00 (approximately 0.1 N HCl). Designations are the same as in Fig. 1. The separation of the two steps is very distinct here too, though somewhat smaller than in Fig. 1. The observed points fit into the calculated curve without any extrapolation being necessary.

The experiments with oxyphenazine are shown in Figs. 1 to 6 and summarized in Fig. 7. For those not accustomed to the methods applied it may be interesting to know that the total amount of dye consumed for all experiments, including the pre-

liminary trials and the losses by filtering off the undissolved residues of the difficultly soluble substance in each individual experiment, amounted to no more than 25 to 30 mg. Figs. 1 to 4 show the step formation at low pH ranges. The smaller the pH, the more distinctly are the steps separated. In Fig 5, steps are no

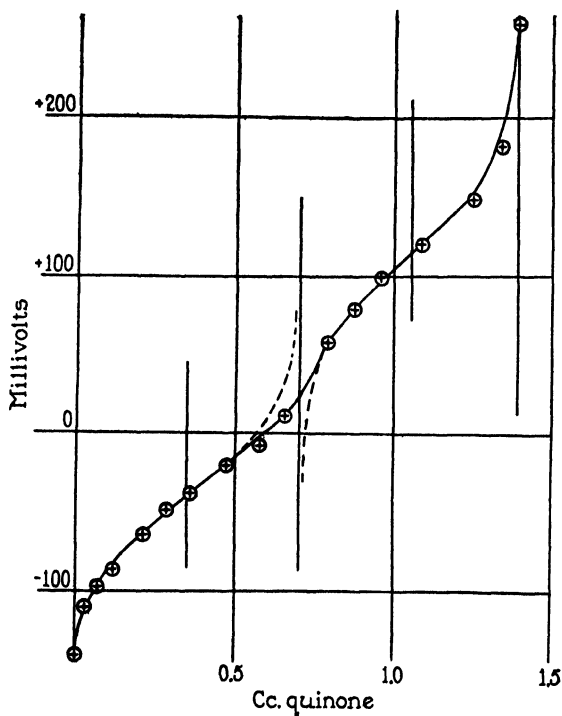


FIG. 3. α -Oxyphenazine at pH 1.858 (the substance is dissolved in about 1.5 cc. of 0.1 M HCl and the volume made up to 15 cc. with 0.1 M KCl solution). Designations are as in Fig. 1. The two steps of potential are here less distinctly separated so that in the middle part the observed curve can be imagined as an overlapping of the two theoretical curves.

longer separable, only the titration curve as a whole is somewhat too steep compared with a regular curve. The drawn out lines in Figs. 1 to 4 are those calculated for two not overlapping titration steps, each with an electron number of 1. The normal potential (*i.e.* the potential of the system in a half oxidized state), separately

shown for each of the two steps, is indicated by perpendicular lines halving each. In Fig. 6 it is shown that the titration curve is the same in every respect whether the original solution of the dye is titrated, or whether this solution is diluted three times. In the case of a meriquinone formation, the curves should differ in their level by 14 millivolts.

In the summarizing figure, Fig. 7, the normal potentials are plotted against pH. Between pH 2 and 9 the curve is rectilinear

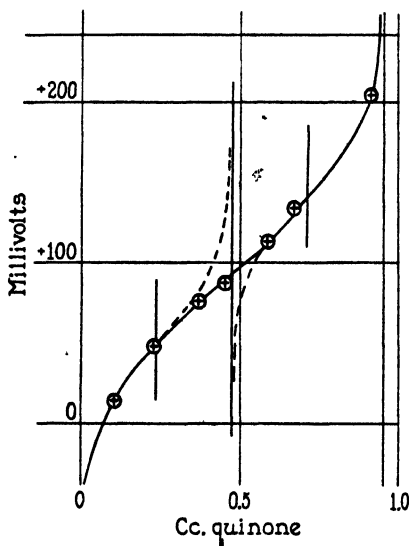


FIG. 4. α -Oxyphenazine at pH 2.640 (1 cc. of 1 M lactic acid and 20 cc. of 1 M sodium lactate). Designations are as in Fig. 1. Here the separation of the two steps is much less distinct; the overlapping is more pronounced. Yet the whole curve cannot be interpreted as an ordinary one step curve because the slope of the curve, taken as a whole, is much too steep.

with the usual slope of 0.06 volts per unit of pH. To the left-hand, the curve branches off, one branch being steeper, the other being flatter than before. The steeper branch has, in general, a slope of 0.09 volts per pH unit. But this branch is not simply a gradual shifting of the 0.06 slope. Rather there is undoubtedly a kind of soft bayonet or "S" form developed. The 0.06 slope leads, to the left-hand side, for a very short interval to a steeper

one, probably to 0.12 slope and then flattens a little to the 0.09 slope. One might be inclined to claim that this part of the curve should have been studied in even smaller pH intervals. There is, however, a difficulty in utilizing data closer together. In this part the overlapping of the two steps in each individual titration curve is so considerable, that the two normal potentials could be

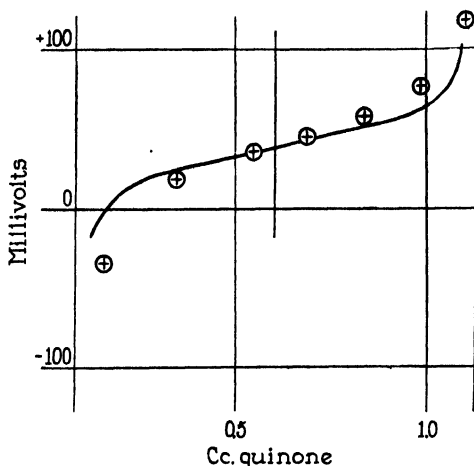


FIG. 5. α -Oxyphenazine at pH 3.472 (4 cc. of 1 M lactic acid + 2 cc. of 1 M sodium lactate, diluted to a volume of 20 cc.). Designations are as in Fig. 1. The drawn out curve, however, is here the one calculated for an oxidation in one step with the electron number 2. The overlapping of the two levels of oxidation is here so great that the only deviation of the observed points from the calculated curve is the fact, that the observed points form a curve just a little steeper than the drawn out curve. All titration curves at pH greater than in this experiment fit perfectly with the one calculated for a one step oxidation with the electron number 2. Since many of such examples have been shown in the previous papers, no further curves for oxyphenazine are described or plotted in detail.

only roughly estimated. This does not help, however, because only the most exact determinations of these normal potentials could lead to a definite decision as to whether or not the above interpretation of the slope is right. Anyhow, the 0.09 slope is precise, and so is the 0.06 slope; and, furthermore, there can be no doubt that the 0.06 slope does not shift smoothly to the 0.09 slope.

Now it is suggestive to correlate the inflections of the curve (Fig. 7) with the color changes of the dye. We begin with the ordinary, oxidized form of oxyphenazine, which corresponds to the field above the curve and, where the curve is branched, above

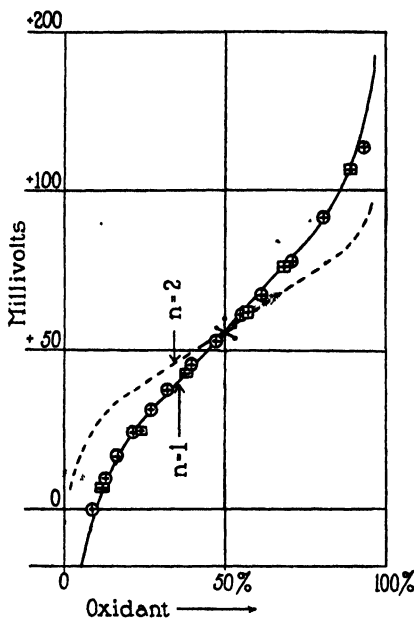


FIG. 6. This shows the independence of the potential of the concentration of the dye. α -Oxyphenazine at pH 1.382 (solution 0.05 M for HCl and 0.1 M for KCl). Only the first step of the oxidation is plotted. The abscissa indicates the amount of quinone, expressed in per cents of that amount which completes the first step of oxidation; the ordinates are potentials. The first experiment is represented by the circles. After finishing this experiment, the whole solution was diluted with the above acid mixture (containing no dye) to a three-fold volume, the dye re-reduced by H_2 + palladium, the hydrogen expelled, and the titration with quinone was repeated. It furnished the points marked with squares. The drawn out line is the one calculated for an oxidation with the electron number 1; the dash line the one calculated for the electron number 2.

the upper branch of the curve. The color is lemon-yellow from pH 2 to almost 8. To the left of 2 it turns over orange to pink, which is fully reached at pH of approximately -1 (HCl much

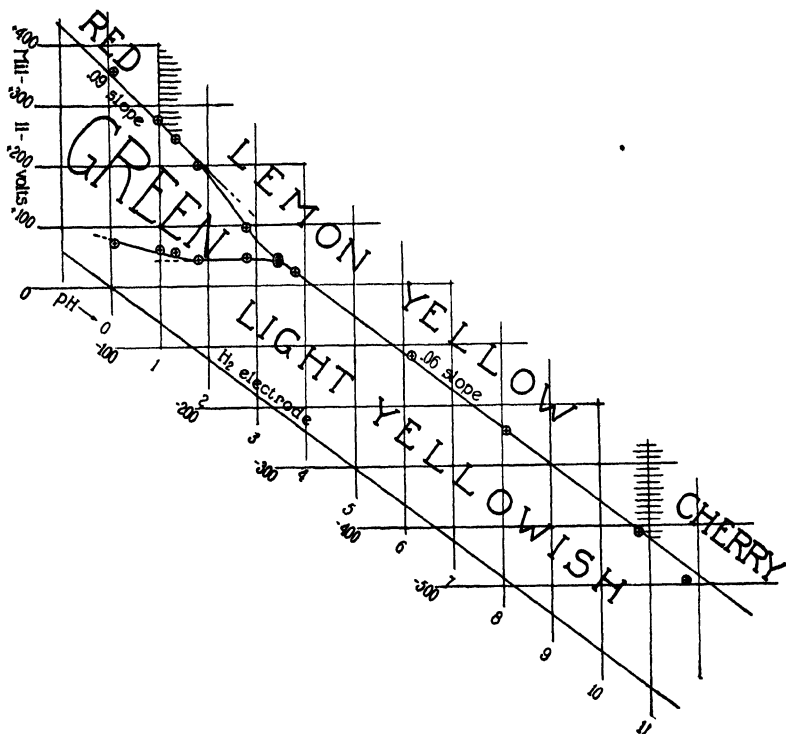


FIG. 7. The normal potential of α -oxyphenazine at varied pH. Abscissa, pH; ordinates, potential of the half reduced system. From pH about 4 to 12 the dye behaves as an ordinary organic dyestuff. The potentials plotted here are those of the half reduced state. At pH < 4, two levels of potential can be distinguished. The potentials plotted are here the potential of the half reduced state of each step of oxidation. The point at pH 3.4 corresponds to Fig. 5. The oblong mark of this point means the average value of two steps so close together that a separation into two distinct values cannot be executed with precision.

The color of the dye depends on pH and potential. In the oxidized form, the color varies from red to lemon-yellow to cherry-red according to pH. The transition zones of color are marked with cross-hatching. The completely reduced form is practically colorless, or slightly yellowish in higher concentrations. The wedge between the two branches of the curve is the area of the emerald green semiquinone. It should be noted that the lower branch of the wedge tends to intersect the hydrogen potential. The point of intersection, however, cannot be attained because of the enormous concentration of hydrogen ions at which it might be expected to occur.

stronger than 1 normal). To the right of pH 10.5 it turns over raspberry color to deep purple, which is fully reached in the pH range of a NaOH solution. There can be little doubt that the turning of the color around pH 0.5 signifies the ionization of one of the N groups, and the one around pH 11 indicates the ionization of a hydroxyl group which may be imagined to be present in the tautomeric Formula IV. The lemon-green color between pH 2 and 9 is the one of a broad isoelectric zone of the dye. It is noteworthy that during the inflections of the curve around pH 2 and 3 no change in color occurs. Neither can another step of dissociation be imagined beside the two mentioned. It can be inferred herefrom that the principles elaborated by Clark and Cohen (16) interpreting bendings in the curves in terms of dissociation constants cannot be simply applied to such a case. No explanation for these bends shall be offered now. This problem is worth a special study and, being of no importance for the subject of this paper, will not be discussed here.

The intermediary step of reduction as framed by the wedge formed by the branches of the curve is always deep emerald green. The unsaturated condition of the nitrogen has a bathochromic effect. The completely reduced form, covering the field below the curve, is always colorless (or very slightly yellowish when in higher concentrations).

In the case of pyocyanine the transition zone of the oxidized form of the dye from blue to red (pH 4.9) did not agree with the beginning of the branching (pH 5.8) nor did it manifest itself by a bending of the curve, as one sees from Fig. 7 in the previous paper (1).

A special discussion, furthermore, is required for the experiment in Fig. 1, at pH 0.08. As one sees from the summarizing Fig. 7 the normal potential of the first step of oxidation is here rather close to the potential of hydrogen of 1 atmosphere pressure at the same pH. Under this condition, the reduction of the dye by palladium and hydrogen of 1 atmosphere pressure cannot be strictly complete. According to the value of this potential as plotted in Fig. 7, the reduction can go on only to 88 per cent. In fact, the reduction by hydrogen gas plus palladium did not proceed to completeness and a residue of the green color of the intermediary state remained permanently. When the hydrogen was

bubbled out and replaced by nitrogen, the green became even more obvious. Here the change of potential, as produced from the mere diminution of the hydrogen pressure, overlaps with the titration curve of the dye. Therefore, the very first steps of the titration curve had to be performed somewhat hastily until steady conditions were reached. In order to evaluate the normal potential as precisely as possible, the actual titration curve of the first step had to be replaced by an idealized curve with no overlapping with the hydrogen potential. This idealization could be per-

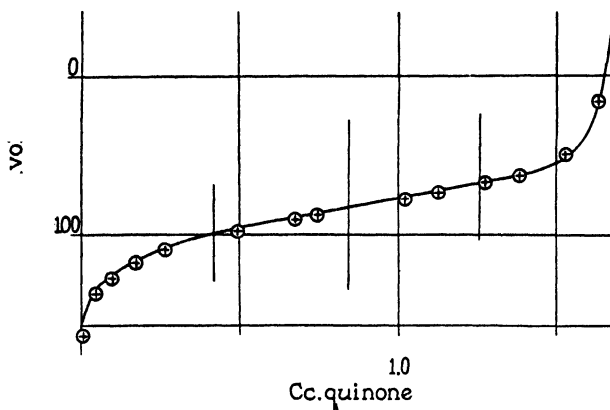


FIG. 8. Rosinduline, dissolved in lactate buffer, pH 3.821, completely reduced, and titrated with quinone. Abscissa, cc. of quinone solution; ordinate, potential, referred to the normal hydrogen electrode. The drawn out curve is the one calculated for an electron number 2, such as in any ordinary dye of the quinoid type.

formed in the following way. The second step of the titration is without such objections, and should use the same amount of oxidant as the first. So we can infer from this second step how many cc. of the oxidant should have been necessary for the first step if the overlapping had not occurred. By this extrapolation the idealized drawn out curve as shown in Fig. 1 was obtained, and the normal potential computed graphically from this theoretical curve. It may be added that this extrapolation is very small, and that even an appreciable error in the graphic construction of this curve would involve an error only of a few millivolts for the value of the normal potential. One has, besides, to take into

consideration that in this range of pH the pH determination itself might not be absolutely strict on account of the liquid junction potential between so acid a solution and the KCl bridge.

No titration curve is printed for any pH > 4. They fitted precisely the theoretical curve for a regular dye such as shown in several preceding publications.

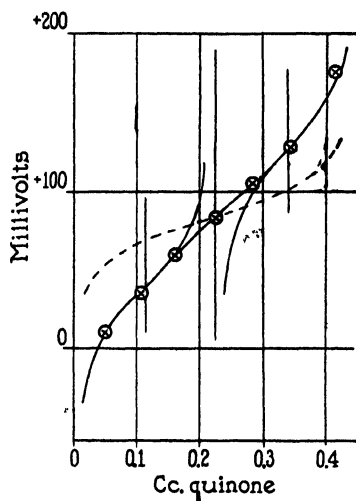


FIG. 9. Rosinduline dissolved in approximately 0.1 N HCl, pH 1.081. Abscissa and ordinates are as in Fig. 8. The dash line curve is the one calculated for the electron number 2; it does not fit at all. The drawn out curve is composed of two half curves, each calculated for the electron number 1, the overlapping part is graphically smoothed so as to form one single curve. In this case, the step formation is graphically not so clear. The whole curve might have been interpreted as a single step curve with the electron number 1. This interpretation, however, would not account for the fact that the color goes from colorless⁴ through violet to orange, the violet being most intense and most pure at the mid-point of the titration.

For rosinduline, Figs. 8 to 10 show some individual titration curves. For the curves at higher pH, Fig. 8 is an example. The drawn out curve is calculated for the electron number 2. Figs. 9 to 11 show the formation of the two steps in acid solutions. Fig.

⁴ The completely reduced form is sometimes designated as colorless, sometimes (as in Fig. 11) as light yellow. It is, in fact, so light in color that in so low a concentration as used in the experiment, Fig. 9, it appears colorless.

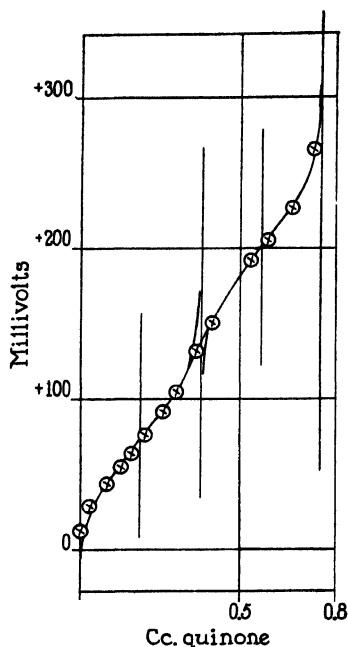


FIG. 10. Rosinduline, dissolved in approximately 1.0 N HCl, pH 0.080. Designations are as in Fig. 9. Here the step formation is already distinct. The two halves of the curve are calculated each for the electron number 1. The color, during the titration, turns from colorless over violet to orange.

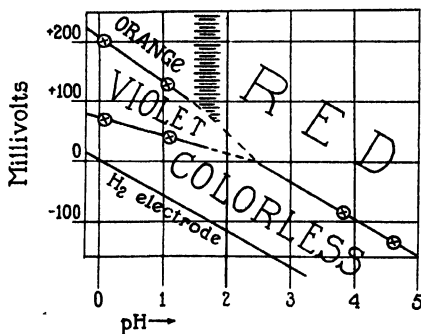


FIG. 11. Normal potential of rosinduline at varied pH. Abscissa, pH. The abscissa is drawn only from pH 0 to 5. An extension of this plot is the diagram Fig. 1 in the previous paper (7). Ordinate, potential of the half reduced dye, referred to the normal hydrogen electrode. The oxidized form (above the curve) is red, respectively orange, according to pH; the transition being marked by dashes. The intermediary form is violet, the reduced form very light yellow.⁴

11 summarizes the results. It may be mentioned that the point for pH 3.82 (Fig. 8) (lactate buffer) has been added after the previous communication (7) and fits in the summarizing diagram (Fig. 11) precisely into the 0.06 slope which this dye shows from very alkaline up to very acid solutions until finally the branching of the slope starts at pH about 2.5. Fig. 11 summarizes the results only for acid solutions, because the slope for higher pH has previously been published in Fig. 1 of a previous paper (7).

SUMMARY

For three dyestuffs, pyocyanine, α -oxyphenazine, and rosinduline, it is shown that between the oxidized and the reduced state there exists an intermediary state, provided the solution is very acid. This intermediary state is recognizable by its particular color. It has been proved by an analytical treatment of the titration curves that this intermediary state is not a meriquinone (a molecular compound of the reduced and the oxidized forms) but a non-polymerized, half reduced state with the character of a free radical. This intermediary state may be designated as a semiquinone, in distinction to a meriquinone. The conditions for its coexistence in a true equilibrium with the other forms of a quinone-hydroquinone system are discussed. This existence depends both on pH and on the oxidation-reduction potential of the solution. These two variables being used as coordinates, the existence of the semiquinone is determined by a wedge-shaped area.

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THE TITRATION OF HYDROXY ORGANIC ACIDS IN THE PRESENCE OF FERRIC AND CUPRIC SALTS

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(Received for publication, April 22, 1931)

In previous work (1, 2) it was pointed out from theoretical reasoning that when a hydroxy organic acid combines with a metal such as iron to form an unionized compound the acid properties of the alcoholic hydroxyl group must be increased. There is abundant evidence in the literature which indicates that the hydrogen of such hydroxyl groups may be replaced by a metal. References to most of this work may be found in the handbooks of Gmelin (3) and Abegg *et al.* (4). There is apparently no direct evidence, however, showing how strongly acidic these groups become. The only titration curves with which the writer is familiar are those of Wark and Wark (5) on the titration of the normal copper salts of several such acids with sodium hydroxide. These authors followed the potential of their solutions during the titration by means of a hydrogen electrode. It is apparent from the potential values that they obtained, that they were not measuring pH but an oxidation-reduction potential instead. The present paper is the result of an attempt to follow, by means of a glass electrode, the titration of representative hydroxy organic acids alone and in the presence of ferric chloride and of cupric chloride.

The glass membranes were made in the manner described by MacInnes and Dole (6). The glass used was obtained from the Corning Glass Company, their No. 015. The MacInnes type of permanently sealed electrode containing 0.1 M HCl and a Ag, AgCl wire was used. The membranes were calibrated against buffers of known pH and this calibration was checked at frequent intervals. The electromotive force was measured by means of a

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vacuum tube potentiometer arrangement of the type described by Partridge (7). The readings obtained were accurate to 1 millivolt.

The results are presented in Figs. 1 to 6. Curves are included for ferric chloride, for cupric chloride, for each of the following acids, lactic, glycollic, oxalic, malic, tartaric, and citric, and for each of

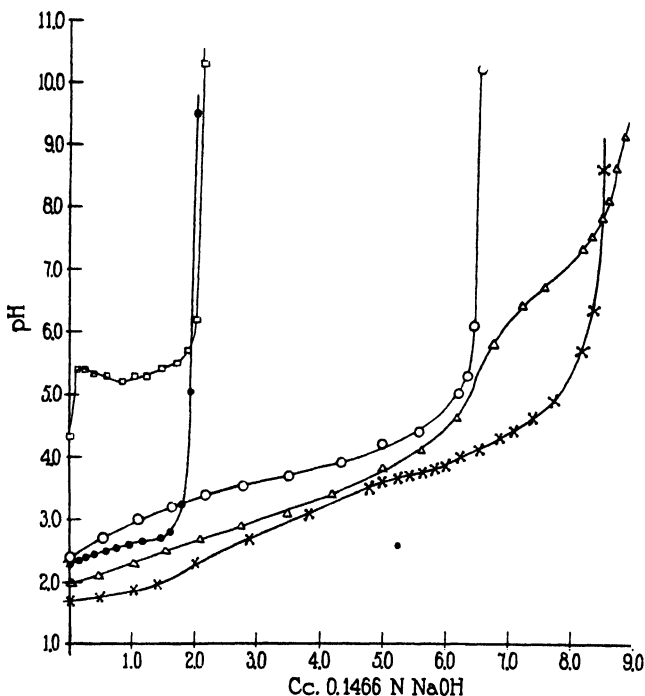


FIG. 1. □ indicates the curve for 1.0 cc. CuCl_2 , ● 1.0 cc. FeCl_3 , ○ 5.0 cc. lactic acid, △ 5.0 cc. lactic acid + 1.0 cc. CuCl_2 , × 5.0 cc. lactic acid + 1.0 cc. FeCl_3 .

these acids in the presence of ferric chloride and in the presence of cupric chloride.

It may be seen from each of the figures that the mixtures of acid and metal chloride are considerably more acidic than either of these substances alone. The beginning of each curve where iron is present clearly indicates that we are titrating some strong

acid. We interpret this as an indication of the fact that the metal and the acid combine to form a complex with the liberation of free hydrochloric acid.

Let us examine the results with lactic acid (Fig. 1) in some detail. It may be seen that the quantity of cupric chloride used requires 2.0 cc. of base for its neutralization. At this point all the copper has been precipitated as cupric hydroxide. The

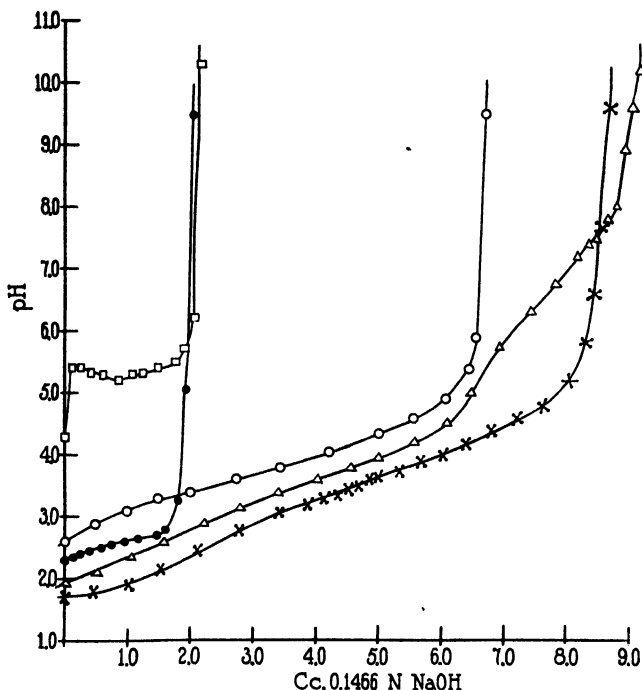


FIG. 2. □ indicates the curve for 1.0 cc. CuCl_2 , ● 1.0 cc. FeCl_3 , ○ 5.0 cc. glycollic acid, △ 5.0 cc. glycollic acid + 1.0 cc. CuCl_2 , × 5.0 cc. glycollic acid + 1.0 cc. FeCl_3 .

quantity of lactic acid used requires 6.5 cc. of base for its neutralization. Now, if the cupric chloride and lactic acid are mixed and the mixture is titrated it requires an amount of base equal to the sum required for the two separate solutions. At this point the copper has again been precipitated as cupric hydroxide. Thus, under the conditions of these experiments lactic acid is unable to prevent

the precipitation of copper as the hydroxide in a neutral or alkaline solution. If we add to the lactic acid an amount of ferric chloride equivalent to the cupric chloride previously added we get a quite different curve. Again the end-point comes when an amount of base equivalent to the two separate solutions has been added, but at this point there is no precipitation of ferric hydroxide. It

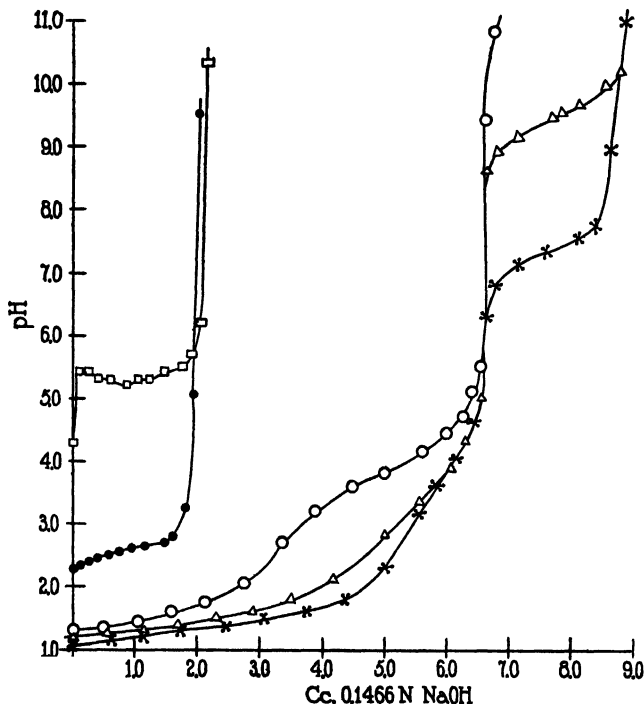


FIG. 3. □ indicates the curve for 1.0 cc. CuCl_2 , ● 1.0 cc. FeCl_3 , ○ 5.0 cc. oxalic acid, Δ 5.0 cc. oxalic acid + 1.0 cc. CuCl_2 , × 5.0 cc. oxalic acid + 1.0 cc. FeCl_3 .

should be noticed that there is a distinct break in this curve at a point corresponding to pH 3.5. The significance of this break will be discussed later.

The results with glycollic acid are presented in Fig. 2. In general they are the same as those with lactic acid. The break in the curve for acid plus iron is less marked but still definitely perceptible.

It may be well to examine, at this point, the curves obtained with some acid that forms a complex with metals, but which does not possess an alcoholic hydroxyl group. Such an acid is oxalic. Fig. 3 presents the curves obtained with it. It may be seen that the first end-point occurs at the end-point of the oxalic acid itself and this is followed by the precipitation of the ferric or cupric

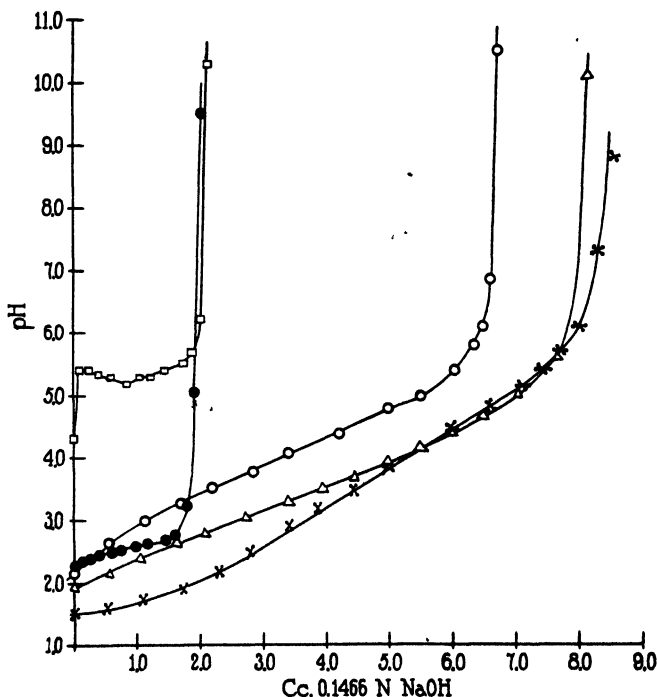


FIG. 4. □ indicates the curve for 1.0 cc. CuCl_2 , ● 1.0 cc. FeCl_3 , ○ 5.0 cc. malic acid, △ 5.0 cc. malic acid + 1.0 cc. CuCl_2 , × 5.0 cc. malic acid + 1.0 cc. FeCl_3 .

hydroxide. Under the conditions of these experiments this acid does not prevent the precipitation of either ferric or cupric hydroxide in an alkaline solution. An important point to be observed is that that part of the curve which corresponds to the second constant of the oxalic acid lies in a much more acid range when either iron or copper is present than in their absence.

Figs. 4 to 6 present the results obtained with malic, tartaric, and citric acids, respectively. It may be observed that with these acids the behavior of iron and of copper is similar. In each case the amount of base required to titrate a mixture of acid plus metal chloride is very considerably more than that required for the acid alone and in no case is there any precipitation of metal

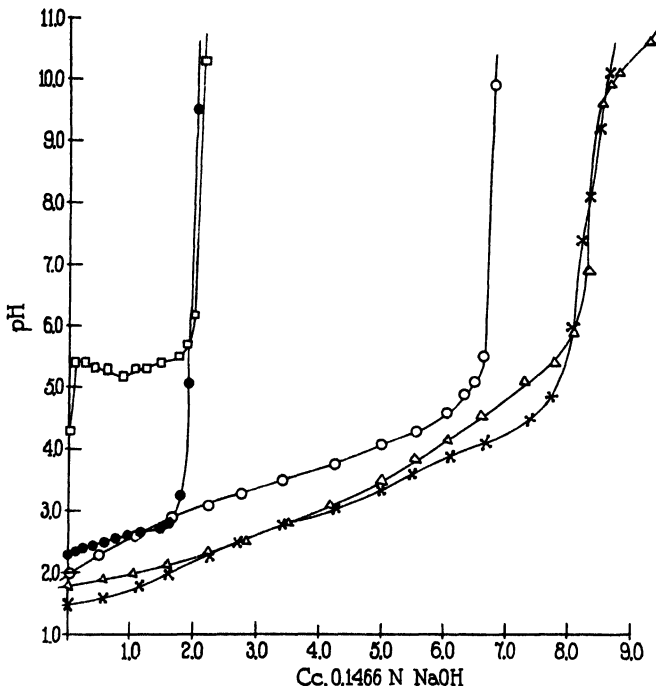


FIG. 5. □ indicates the curve for 1.0 cc. CuCl_2 , ● 1.0 cc. FeCl_3 , ○ 5.0 cc. tartaric acid, Δ 5.0 cc. tartaric acid + 1.0 cc. CuCl_2 , × 5.0 cc. tartaric acid + 1.0 cc. FeCl_3 .

hydroxide. It should also be noted that in no case does the titration of such a mixture require an amount of base as large as the sum required for the titration of the two solutions separately.

DISCUSSION

Let us consider the results with lactic acid first. We saw that the mixture of lactic acid and ferric chloride required for its

neutralization an amount of base equal to that required for the two solutions separately. When the solutions are titrated separately all of the iron is precipitated as ferric hydroxide, but when the mixture is titrated no precipitate is formed. The question is, how did we use up so much base without precipitating any ferric hydroxide. There are at least two possible answers. The

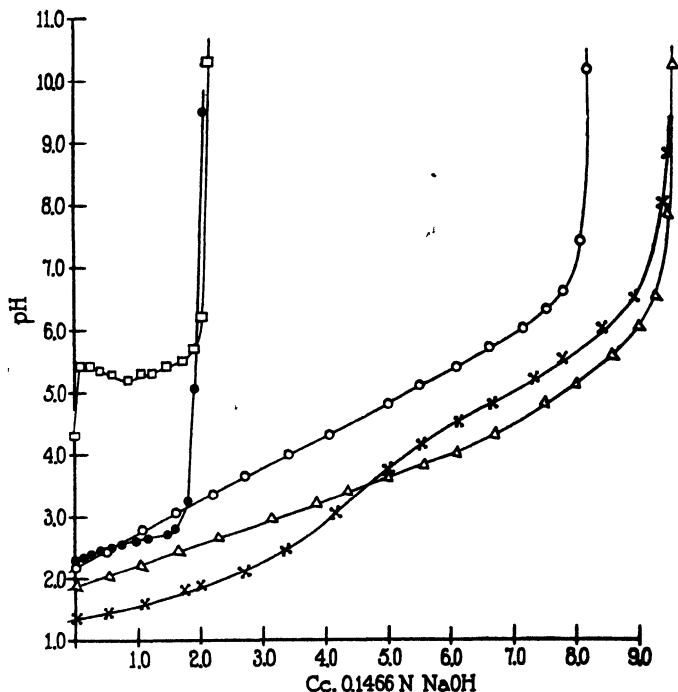


FIG. 6. \square indicates the curve for 1.0 cc. CuCl_2 , \bullet 1.0 cc. FeCl_3 , \circ 5.0 cc. citric acid, \triangle 5.0 cc. citric acid + 1.0 cc. CuCl_2 , \times 5.0 cc. citric acid + 1.0 cc. FeCl_3 .

first of these is that ferric hydroxide was really formed, but it remained in colloidal solution instead of precipitating. One fact seemingly in favor of this view is that these solutions are not readily diffusible through a collodion bag. However, if one examines the various compounds reported by Gmelin (3) another explanation suggests itself for this lack of diffusibility. This is that the compounds which exist in solution have high molecular

weights. Many of those reported contain 3 or more iron atoms and a corresponding number of organic residues. We have tested the diffusibility of the alkaline solutions of iron with each of the acids studied. In each case it is very slow, but in each case some iron passes through the collodion bag, in contrast to the complete lack of diffusibility with an ordinary sol of ferric hydroxide.

There are other difficulties in the way of accepting this colloidal explanation. These solutions are not sensitive to electrolytes. Any one of them may be saturated with ammonium sulfate without producing any precipitate. Relatively large amounts of calcium chloride do produce a precipitate, but this precipitate is not ferric hydroxide. It would seem to be the calcium salt, analogous, in the case of lactic acid, to the sodium, potassium, and ammonium salts isolated by Hofmann (8) to which he assigned the formula $M[\text{Fe}(\text{CH}_3\text{CHOC}_2)_2]$, where M represents either sodium, potassium, or ammonium.

If these solutions contain the iron as colloidal ferric hydroxide then we have the problem of determining why an alcoholic hydroxyl group is so very important for the maintenance of the colloidal state. Thus lactic acid and glycollic acid would be good peptizing agents whereas propionic acid and acetic acid are of no avail. Similarly, neither oxalic acid nor succinic acid can produce the necessary action, but malic acid works very nicely and tartaric acid is exceedingly effective.

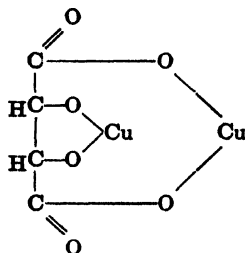
The other explanation is that when the lactic acid and iron combined to form a complex the alcoholic hydroxyl group took part in this combination. Such an action must make the oxygen of this group more positive and as a result the hydrogen must become more acidic. Then to the extent that iron is present lactic acid will behave as a dibasic acid. This will account nicely for the amount of base used without making it necessary to assume the formation of ferric hydroxide. Furthermore, it will account nicely for the two steps in the titration curve for lactic acid plus ferric chloride. According to this interpretation the pK_a for this alcoholic hydroxyl group is about 3.85. We may point out that this interpretation is in accord with our previous contention (2) that in dehydrogenation reactions the hydrogen separates from the molecule as hydrogen ion and the process of activation is simply a process of increasing the acid dissociation constant of the group involved.

An interesting observation concerning the acidity of such hydroxyl groups has recently been reported by Hölzl (9). He found that if the ester of salicylic acid was dissolved in absolute alcohol the hydroxyl group then exhibited appreciably acid properties.

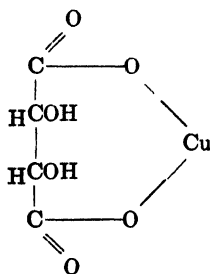
The explanation given for the results obtained with lactic acid will apply equally well to those obtained with glycollic acid. Let us see now how it will apply to oxalic acid. If this free acid forms an unionized compound with iron, and the curves (Fig. 3) show that it does, we would expect that the OH group of the undissociated carboxyl would take part in the combination. The result should be that this second carboxyl group would become a stronger acid than it is in the absence of a metal. The curves show that this is markedly true.

The same general explanation will apply to the results with malic, tartaric, and citric acids. Here we have the added complication, however, that the amount of base required for the neutralization of a mixture of any one of these acids with ferric or cupric chloride is not quite as large as the sum required for the neutralization of the two separate solutions. The explanation for this must lie in the formation of complex basic salts. This is in agreement with the results obtained by Packer and Wark (10) and by Wark and Wark (5) in their work on copper tartrates, as well as with the results of many other investigators (3, 4). Dumanskii and Chalisew (11), however, regard these copper tartrates as colloidal solutions of copper hydroxide. Similarly, Dumanskii and Yakovlev (12) regard solutions of ferric iron and various hydroxy acids as colloidal solutions of ferric hydroxide.

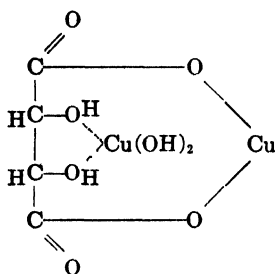
We may inquire here just what the difference is between the colloidal and the non-colloidal view-point regarding these solutions. Jellinek and Gordon (13) have obtained a crystalline copper salt of copper tartrate. They assigned to it this formula:



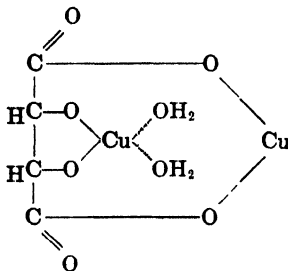
Dumanskii and Chalisew (11) have prepared what they believe is an identical salt and they assign to it this formula: $\text{Cu}T \cdot \text{Cu}(\text{OH})_2 \cdot \text{H}_2\text{O}$. The T indicates tartrate. Let us see how this differs from Jellinek and Gordon's formula. We may write the $\text{Cu}T$ as



To this is attached a $\text{Cu}(\text{OH})_2$ by means of a point (\cdot). We must interpret this point as some kind of an attraction and this attraction must be furnished by the OH groups of the tartrate, for one cannot substitute succinic acid for the tartaric acid. By incorporating this into the formula we have



We may rewrite this as



This now differs from Jellinek and Gordon's formula by 2 molecules of water. If Jellinek and Gordon's compound be taken to represent the non-colloidal point of view and Dumanskii and Chalisew's compound to represent the colloidal point of view then, remembering that basic compounds may also be formed in which there is one copper attached to each hydroxyl group, and that one copper may be attached to a hydroxyl group in each of 2 tartrate molecules, there is really no difference as far as present data are concerned between the two views.

The application of these results to the titration of any one or any mixture of the hydroxy acids considered is clear. If one were to determine by titration the amount of these acids present in a solution containing proportionately as much iron, or in some cases as much copper, as the solutions used here, and were to use the turning point of phenolphthalein as end-point, the result would be in error by over 30 per cent.

SUMMARY

Titration curves, obtained by means of the glass electrode, are reported for lactic, glycollic, oxalic, malic, tartaric, and citric acids, and for each of these acids in the presence of ferric chloride and in the presence of cupric chloride.

It is shown that the presence of these metal salts greatly alters the titration curves. The nature of this change is discussed and the application of the results is pointed out.

It is a pleasure to acknowledge my indebtedness to Dr. L. Michaelis, in whose laboratory this work was carried out, for his generous assistance and interest in this work.

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THE METABOLISM OF THE PHOSPHOLIPIDS

III. THE COMPARATIVE INFLUENCE OF VARIOUS FATS ON THE DEGREE OF UNSATURATION OF THE PHOSPHOLIPIDS AND NEUTRAL FAT IN THE TISSUES OF THE RAT

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(Received for publication, April 27, 1931)

INTRODUCTION

In the first paper of this series (Sinclair, 1930; *a*) data were presented which seemed to prove beyond a reasonable doubt that the degree of unsaturation, and therefore, the composition, of the phospholipids in various tissues of the cat is by no means constant but, on the contrary, is markedly influenced by the type of diet fed. However, the diets used (beef kidney and beef muscle) were of such a complex composition that it was impossible to arrive at any definite conclusion as to the relationship which exists between the character of the food fat and the composition of the tissue phospholipids. Consequently, it was decided to investigate specifically the comparative influence of diets, either essentially devoid of fat or containing one of several fats of widely different compositions, on the degree of unsaturation of the phospholipid fatty acids in the tissues of the rat. The necessity for further investigation of the influence of food fat on tissue phospholipids became the more apparent with the publication of a paper by Terroine and Hatterer (1930) in which they reaffirmed the conclusion of Terroine and Belin (1927) that both the composition and the amount of the phospholipid in a tissue are entirely uninfluenced by diet.

EXPERIMENTAL

Procedure—In conformity with the procedure employed in previous work, the iodine number of the fatty acids obtained by saponification of the acetone-insoluble lipids has been used as an

index of the composition of the tissue phospholipids. As will be pointed out subsequently in some detail, the fatty acids are always contaminated by a peculiar white substance, presumably a mixture of unsaponified sphingomyelin and cerebrosides. Consequently, the iodine numbers as given in Tables II and III are

TABLE I
Percentage Composition of Diets

Ingredients	Fat-free Diet 20	Unpurified		Fat-containing	
		Diet 1	Diet 3	Diet 260	Diet 262
	<i>per 100 gm.</i>	<i>per 100 gm.</i>	<i>per 100 gm.</i>	<i>per 100 gm.</i>	<i>per 100 gm.</i>
Casein.....	21.9	21.9	17.1	27.3	21.8
Cane-sugar.....	65.8	65.8	70.3	38.1	41.0
Salt mixture*.....	3.6	3.6	3.8	4.4	4.8
Fat.....				19.3	21.3
Yeast.....	8.7	8.7	8.8	10.9	11.1
	<i>per 100 calories</i>	<i>per 100 calories</i>	<i>per 100 calories</i>	<i>per 100 calories</i>	<i>per 100 calories</i>
Casein.....	23.3	23.0	18.0	23.1	18.0
Cane-sugar.....	69.8	69.0	74.0	32.2	34.0
Fat.....				36.6	40.0
Yeast†.....	6.9	8.0	8.0	8.1	8.0

Vitamins A and D were supplied by feeding the unsaponifiable matter from cod liver oil. In Diets 20, 1, and 260, 10 mg. of oscodal per gm. of yeast were added to the ration. Each rat on Diet 3 received daily by mouth the unsaponifiable matter from 150 mg. of cod liver oil dissolved in 2 drops of liquid petrolatum. In Diet 262 the unsaponifiable matter from 5 gm. of cod liver oil was dissolved in each 100 gm. of fat.

The oscodal was kindly supplied by the H. A. Metz Laboratories, Inc., through the courtesy of Dr. H. E. Dubin.

* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

† Calculated on the basis of 75 per cent assimilable protein and carbohydrate and 5 per cent fat.

somewhat too low. Fortunately the amount of this contaminating substance present in the phospholipid fatty acids seems to be fairly constant so that the comparative significance of the data is not vitiated.

Data were obtained on the iodine numbers of the phospholipid fatty acids from the entire bodies, the carcasses,¹ and the livers of

¹ The carcass comprises the bony skeleton and its musculature.

rats of both sexes ranging in weight from about 75 up to 260 gm. The procedure followed in extracting and separating the lipids has been given in detail in the second paper of the series (Sinclair, 1930, *b*). Latterly, in order to insure against possible contamination of the phospholipids by neutral fat, precipitation of the phospholipids from ether solution by acetone has been performed three times.

Diets—The composition of the diets used is given in Table I.

The diet designated as fat-free was not strictly so. By analysis of the extracted casein and yeast, it has been found that there was

TABLE II

Influence of Food Fat on the Degree of Unsaturation of the Phospholipid Fatty Acids of the Rat

Diet	Entire animal		Carcass		Liver	
	No. of determinations	Average Iodine No.	No. of determinations	Average Iodine No.	No. of determinations	Average Iodine No.
Fat-free.....	13	101.2 \pm 4.2				
Unpurified, poor in fat...	4	99.4 \pm 3.6	9	100.8 \pm 2.8	6	124.4 \pm 3.9
Coconut oil, I.N. 10*.....	3	114.4 \pm 1.8	4	124.1 \pm 4.7	2	142.8 \pm 1.2
Butter, I.N. 30.....	3	118.8 \pm 0.4	6	124.7 \pm 4.0	2	149.8 \pm 0.3
Lard, I.N. 68.....	3	126.4 \pm 2.7	6	137.4 \pm 2.4	3	157.9 \pm 4.1
Olive oil, I.N. 83.....	7	124.6 \pm 2.2	6	133.8 \pm 1.4	3	151.3 \pm 6.2
Cod liver oil, I.N. 165.....	9	145.5 \pm 2.4	7	160.4 \pm 2.2	3	184.2 \pm 2.1
Linseed oil, I.N. 177.....	3	125.6 \pm 1.6	6	137.0 \pm 1.7	3	156.1 \pm 4.0

* I.N. represents iodine number.

present about 0.25 gm. of fatty material per 100 gm. of mixed diet, the most of the fat being present in the extracted yeast. In the unpurified diet, which was of the same composition as the fat-free, neither the casein nor the yeast was extracted. Analysis of the ingredients has shown that this diet contained on the average 0.64 gm. of fatty acids per 100 gm. of mixed ration, the average iodine number of these fatty acids being 59.

Both food and tap water were provided *ad libitum*.

Results—The iodine numbers of the phospholipid fatty acids from the entire bodies, the carcasses, and the livers of rats raised

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on the various diets are given in Table II. The standard deviation of the individual results from the mean has been calculated by use of the formula $\sigma = \sqrt{\frac{\sum x^2}{n}}$, in which σ is the standard

TABLE III
Detailed Data on Iodine Numbers of Phospholipid and Neutral Fat Fatty Acids from Carcasses of Rats Fed on Diets Containing Butter, Olive Oil, and Linseed Oil

Fat present in diet	Rat No.	Age	Body weight	Period on diet	Iodine No.	
					Phospho-lipid fatty acids	Neutral fat fatty acids
		<i>days</i>	<i>gm.</i>	<i>days</i>		
Butter Diet 260	J, 5b-1 ♀	41	76	11	131	60
	J, 5b-2 ♀	73	122	44	123	61
	J, 5b-3 ♀	107	164	77	124	58
	" 262 J, 5b-4 ♂	91	214	50	121	52
	J, 5b-5 ♂	94	222	57	120	53
	J, 5b-6 ♂	96	206	62	129	53
Olive oil Diet 260	J, 2b-1 ♀	69	114	28	131	80
	J, 2b-2 ♀	92	132	51	133	80
	J, 2b-3 ♀	112	136	71	135	81
	" 262 J, 2b-4 ♂	88	238	60	134	81
	J, 2b-5 ♂	88	234	59	135	81
	J, 2b-6 ♂	98	175	63	134	80
Linseed oil Diet 260	J, 4b-1 ♀	51	76	18	135	144
	J, 4b-2 ♀	61	130	28	138	156
	J, 4b-3 ♀	123	148	91	139	141
	" 262 J, 4b-4 ♂	98	250	64	138	143
	J, 4b-5 ♂	98	240	57	137	139
	J, 4b-6 ♂	113	228	76	134	137

deviation, \bar{x} is the deviation of each result from the mean, and n is the number of individual results.

The comparative influence of these various fats on the iodine numbers of both the phospholipid and the neutral fat fatty acids is illustrated by bar diagrams, in which the solid bars represent the

average iodine numbers of the phospholipid fatty acids, and the hatched bars those of the neutral fat fatty acids. Fig. 1 is the bar diagram for the entire bodies of rats, Fig. 2 is that for the carcasses.

In Table III are presented the detailed data for rats raised on diets containing butter, olive oil, and linseed oil in order to show the apparent lack of influence of sex, age, and body weight on the iodine number of the phospholipid fatty acids and also to show more clearly than is brought out by the standard deviation the extent of variation among the individual rats raised on the same diet.

In Table IV are set forth the data pertaining to the percentage amount of contaminating substance found in the phospholipid fatty acids and the iodine numbers of the "purified" fatty acids.

TABLE IV
Showing the Percentage Amount of Contaminating Substance in the Phospholipid Fatty Acids and Its Effect on the Apparent Iodine Numbers of the Latter

Tissue	Range in Iodine Nos. of phospholipid fatty acids	Percentage amount of contaminating substance in phospholipid fatty acids		Percentage difference in I.N. of "crude" and "purified" fatty acids $\frac{\text{I.N.}_p - \text{I.N.}_c}{\text{I.N.}_c} \times 100$	
		No. of determinations	Average	No. of determinations	Average
Entire rat Carcass	98-146	8	10.51 \pm 2.54	8	9.8 \pm 2.0
	93-161	45	8.09 \pm 2.42	43	6.0 \pm 2.5
	93-129	23	8.76 \pm 2.02		
	130-161	22	7.38 \pm 2.46		

DISCUSSION

Presence of Unsaponified Material in Phospholipid Fatty Acids—Throughout our work on the phospholipids of rat tissues it has been invariably observed that a white turbidity develops in the petroleum-ether solution of the phospholipid fatty acids, either during the primary extraction in the separatory funnel, or during the second extraction. While this occurrence was always a source of uneasiness as to the possibility of its being an indication of appreciable oxidation of unsaturated fatty acids, it seemed futile to try to isolate from the small amounts of fatty acids being

handled sufficient substance for identification. However, in the course of time it was discovered that the addition of acetone to the slightly turbid solution of phospholipid fatty acids in petroleum-ether resulted in the development of a flocculent white precipitate which could be centrifuged out. Forthwith, a determination of the percentage amount of the substance present in the phospholipid fatty acids was made each time on an aliquot sample of the fatty acids, since our chief worry was lest the presence of this substance vitiate the comparative significance of the iodine numbers. After removal of the white precipitate, the acetone was evaporated from the "purified" fatty acids and the iodine number again determined. The data obtained have been summarized in Table IV.

The fact of greatest importance to the subject under discussion, namely, the comparative influence of food fats on the composition of tissue phospholipids, is that there is no evidence of any correlation between the amount of this contaminating substance and the degree of unsaturation of the phospholipid fatty acids. Consequently it seemed unlikely that the substance had its origin in oxidative changes in the unsaturated fatty acids. Although the routine method of saponification has been to boil the acetone-insoluble lipids for 4 hours with a 10 per cent solution of NaOH in 50 per cent alcohol, the precipitation of the substance by acetone seemed to indicate that it was unsaponified lipid. In order to determine whether or not longer boiling or different saponifying agents would result in uncontaminated fatty acids, about 6 gm. of acetone-insoluble lipids were divided into four fractions. Fraction 1, boiled for 4 hours with 10 per cent NaOH in 50 per cent alcohol, yielded 11.5 per cent of the substance; Fraction 2, which was centrifuged to remove the ether-insoluble lipids and then was saponified in the same manner as Fraction 1, gave 8.5 per cent; Fraction 3, boiled for 15 hours with 15 per cent KOH in 50 per cent alcohol, gave 4.8 per cent; and Fraction 4, boiled for 15 hours with 5 per cent aqueous H_2SO_4 , yielded 4.2 per cent. While no definite conclusion is yet possible, such information as has been obtained indicates rather strongly that this substance which resists saponification and is extracted with the phospholipid fatty acids is a mixture of sphingomyelin and cerebrosides.

Influence of Food Fat on Composition of Depot Fat—Although the primary object of this investigation was to determine the

influence of food fat on the composition of the tissue phospholipids, at the same time it was an easy matter to obtain information as to the effect of the various fats fed on the degree of unsaturation of the depot fat. The data obtained are presented in the bar

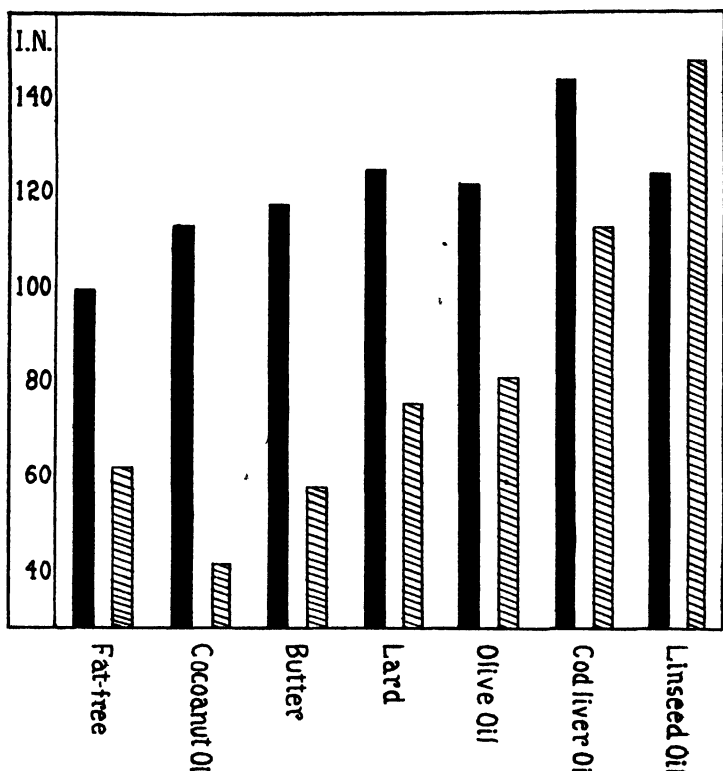


FIG. 1. The comparative influence of various fats on the degree of unsaturation of the phospholipid and neutral fat fatty acids from the entire bodies of rats. The solid bars represent the iodine numbers of the phospholipid fatty acids, the hatched bars those of the neutral fat fatty acids.

diagrams given in Figs. 1 and 2. Little need be said in the way of discussing these data, since they merely corroborate the published observations of Ellis and coworkers (1925, 1926, 1930), Anderson and Mendel (1928), Eckstein (1929), Reed, Yamaguchi, Anderson, and Mendel (1930), and many others.

Nevertheless, there is one point of rather considerable importance which seems to be borne out by these data. It will be remarked that, when the rat is fed on a diet which is practically

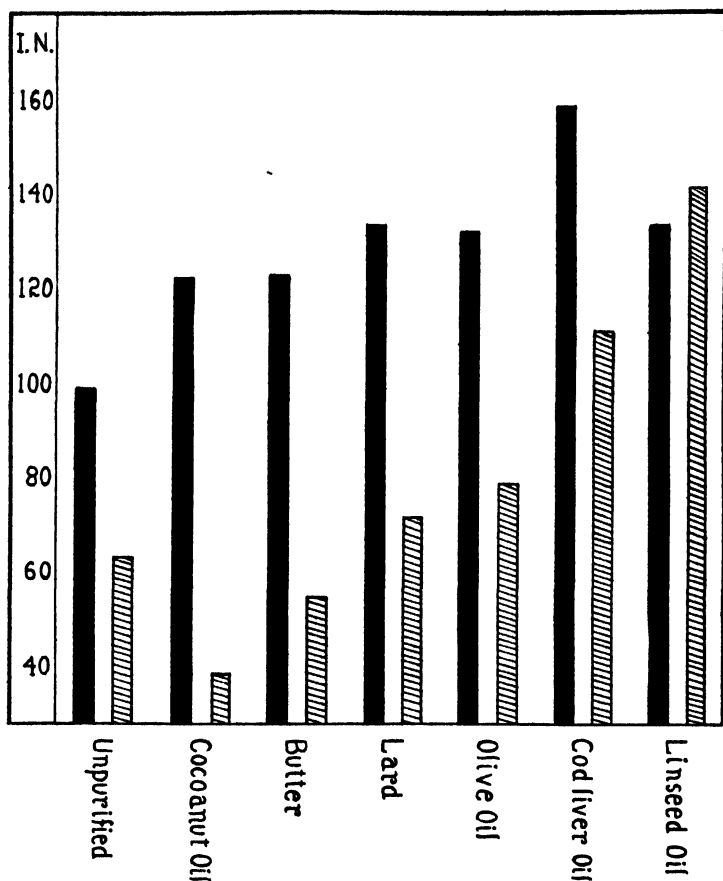


FIG. 2 The comparative influence of various fats on the degree of unsaturation of the phospholipid and neutral fat fatty acids from the carcasses of rats. The solid bars represent the iodine numbers of the phospholipid fatty acids, the hatched bars those of the neutral fat fatty acids.

devoid of fat, the neutral fat deposited in the adipose tissue has an iodine number of about 60. If, however, the rat has been fed a

diet which contains about 20 per cent of fat, the depot fat is neither synthetic fat nor unchanged food fat, nor does the iodine number of the depot fat necessarily lie in between the iodine numbers of synthetic and food fat. The latter may be so, and yet again it may not. For instance, when coconut oil is fed, the depot fat has an iodine number of around 40, that is, between 60 and 10. On the other hand, when lard is fed, of which the iodine number is 68, the deposit fat has an iodine number averaging 77. Olive oil, however, results in the deposition of a neutral fat which has an iodine number only slightly lower than that of olive oil itself. These facts indicate, in our estimation, that the adipose tissue cells exhibit some selection in storing away fat from the blood, presumably by reason of a greater permeability of the cell membranes to oleic acid than to the fatty acids of greater or less unsaturation.

*Influence of Food Fat on Composition of Tissue Phospholipids—*It may be seen from Table II that, when rats are raised on a diet which is practically devoid of fat, the phospholipid fatty acids of both the entire animals and the carcasses have a low degree of unsaturation; in round numbers about 100. If, however, the diet contains a fat, even if it be coconut oil or butter, the tissue phospholipids are more unsaturated than those of rats raised on the fat-free diet. It may also be seen from Table II and Figs. 1 and 2 that there is a total absence of any direct relationship between the iodine number of the food fat and that of the phospholipid fatty acids in the tissues. Thus, lard, olive oil, and linseed oil all result in phospholipids of essentially the same degree of unsaturation, while cod liver oil, with an iodine number less than that of linseed oil, leads to the formation of very highly unsaturated phospholipids. It is apparent from the figures that entirely different relationships exist between the character of food fat and the composition of the tissue phospholipids than exist between food fat and depot fat.

The discussion of the probable significance of the low degree of unsaturation of the phospholipids of rats raised on the fat-free and unpurified diets, of the similarity in the effects produced by lard, olive oil, and linseed oil, and of the outstanding position of cod liver oil will be reserved for a later paper in which will be presented data on the comparative influence of various amounts of the same

fat on the degree of unsaturation of the phospholipids of the rat. Also, it seems advisable to defer discussion of the bearing of the facts presented in this paper on our conceptions of the functional significance of the phospholipids until the publication of a subsequent paper which will deal with the rate of phospholipid metabolism.

In the light of the evidence presented in the first paper of this series (Sinclair, 1930, *a*), and in the present paper, it is difficult at first sight to understand how Terroine and Hatterer (1930) could have arrived at the conclusion that the composition of the tissue phospholipids is not influenced by the nature of the diet. However, closer insight into their paper and our own reveals certain probable explanations. In the first place, the diets used by Terroine and Hatterer, predominantly wheat or hemp-seed, while widely different in the amount of fat which they contained, may not have differed so very much in the composition of that fat. It has been found (unpublished experiments) that small amounts of fat exert proportionately a much greater effect on the composition of the phospholipids than do larger amounts. The essential similarity in the effects exerted by lard, olive oil, and linseed oil brings out forcibly the error which would have resulted if, let us say, lard (iodine number 68) and linseed oil (iodine number 177) had been used in experiments to determine whether or not food fat exerts an influence on the composition of tissue phospholipids. On the other hand, the difference between the average iodine numbers of the phospholipid fatty acids of rats raised on coconut oil (iodine number 10) and olive oil (iodine number 83) is quite significant.

Table II shows that the phospholipid fatty acids from the carcasses are in general more unsaturated than those from the entire bodies of rats raised on the same diets. There is reason to believe that this difference is chiefly due to the comparatively low degree of unsaturation of the phospholipids of the skin. Thus, the few experiments which have been carried out show that the phospholipid fatty acids from the skins of rats raised on these various diets have the following iodine numbers: unpurified 89, coconut oil 98, butter 105, lard 105, linseed oil 103, and olive oil 92. These few experiments indicate also that the phospholipid fatty acids from the skin are contaminated by considerably more

of the unsaponified lipid than is true of the other tissues studied. It has been found, too, that the phospholipid fatty acids from the flayed rat have approximately the same iodine number as those from the carcasses. However, this is largely a coincidence since the data on the iodine numbers of phospholipid fatty acids from the various tissues of the cat (Sinclair, 1930, *a*) and of the beef (Bloor, 1926, 1927, 1928) show definite differences between tissues of the same animal.

SUMMARY

Determinations have been made of the iodine numbers of the phospholipid and neutral fat fatty acids from the entire bodies, the carcasses, the livers, and in a few cases, from the skins of rats raised on diets either very poor in fat or containing one of several common fats.

The phospholipid and neutral fat fatty acids in the tissues of rats raised on a fat-free or fat-poor diet have a comparatively low degree of unsaturation.

When the diet contains a fat, the phospholipid fatty acids are always more unsaturated than those of rats raised on the fat-free diet; the depot fat, on the other hand, may have a lower or higher iodine number than the synthetic fat, depending upon the iodine number of the food fat.

There is no parallelism between the iodine number of the food fat and that of the tissue phospholipids. Coconut oil and butter do result in tissue phospholipids of moderately low degree of unsaturation, but lard, olive oil, and linseed oil exert the same influence, while the feeding of cod liver oil leads to the formation of tissue phospholipids with a very high degree of unsaturation.

There is rough parallelism but not a proportionality between the iodine number of the food fat and that of the neutral fat stored away by the animal.

The author has enjoyed in Professor W. R. Bloor a constant source of encouragement and advice.

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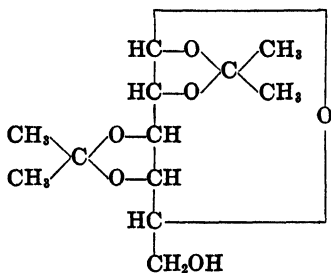
THE RING STRUCTURE OF DIACETONE GALACTOSE

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(Received for publication, May 28, 1931)

The diacetone derivatives of aldohexoses and aldopentoses so far examined all belong to the furanose type with the exception of diacetone arabinose of which the ring structure has not yet been proved by the direct method. Also, in the case of diacetone galactose the ring structure is not yet known. *A priori*, however, the pyranose structure may be expected for diacetone galactose inasmuch as the hydroxyl in position (6) in this derivative was shown by Svanberg and Bergmann¹ and by Freudenberg and coworkers² to be unsubstituted, and also inasmuch as in this case the hydroxyls in positions (3) and (4) offer the most favorable condition for the formation of a strainless 5-membered isopropylidene ring. This may be seen from the following figure.



Indeed, Freudenberg expressed preference for this structure.

Several years ago³ we came into possession of monoacetone

¹ Svanberg, O., and Bergmann, S. W., *Arkiv. Kemi, Mineral. Geol.*, **9**, 3 (1924).

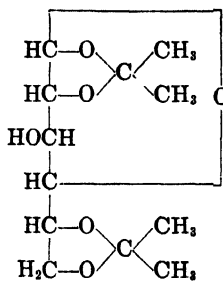
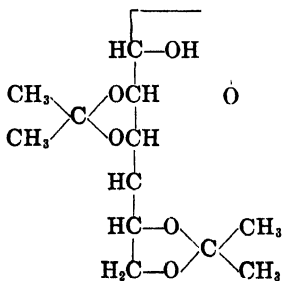
² Freudenberg, K., and Smeykal, K., *Ber. chem. Ges.*, **59**, 100 (1926).
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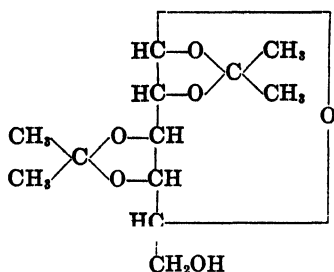
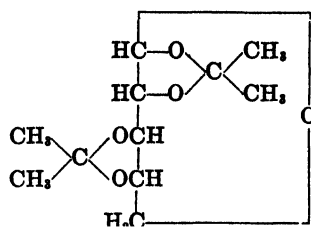
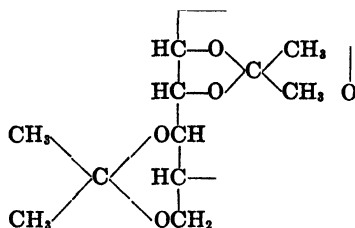
³ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **64**, 473 (1925).

galactose which was formed spontaneously in the preparation of diacetone galactose. However, the quantity then in our possession was insufficient for an exhaustive study of its structure. A somewhat larger quantity of the substance has now been prepared by a process described in the experimental part, and this material was used for the determination of its ring structure. The substance was methylated in acetic solution with dimethyl sulfate and sodium hydroxide. The trimethylmonoacetone-*d*-galactose prepared in this manner was oxidized to the corresponding acid and this converted into the lactone. On the basis of Hudson's rule the <1, 4> lactone should be levorotatory and the <1, 5> lactone dextrorotatory. The one obtained by us had a rotation of $[\alpha]_D^{20} = +46.8^\circ$, thus indicating the <1, 5> ring structure of the lactone. This conclusion has been substantiated by the rate of the lactone formation of the free acid, inasmuch as half equilibrium was reached in 6 hours. Thus it is definitely established that the monoacetone-*d*-galactose belongs to the pyranose type and hence it is certain that diacetone-*d*-galactose likewise belongs to this type.

In view of the analogy in the configurations of galactose and arabinose it may be warranted to assume that diacetone arabinose also belongs to the pyranose type.

By comparison of the diacetone derivatives of three hexoses and of two pentoses, it can be seen that in every case the ring is formed in such a way as to facilitate acetylation.

Diacetone-*d*-glucoseDiacetone-*d*-mannose

Diacetone-*d*-galactoseDiacetone-*l*-arabinoseDiacetone-*d*-xylose

EXPERIMENTAL

Diacetone-d-Galactose—A mixture of 100 gm. of galactose, 200 gm. of anhydrous copper sulfate, 2000 cc. of commercial acetone, and 10 cc. of concentrated sulfuric acid was shaken at room temperature for 18 to 20 hours. The acetone solution was filtered and neutralized with dry calcium hydroxide. The filtered solution was concentrated under diminished pressure to a syrup and distilled. The yield of diacetone galactose boiling at 131–135°, $p = 0.2$ mm., was 96 gm.

Monoacetone-d-Galactose—Large quantities of diacetone galactose, admixed with varying small amounts of hydrochloric acid in acetone, were allowed to stand in the cold room (temperature 10–16°) during the summer months. The best results were obtained when 100 gm. of diacetone galactose were diluted with a small amount of acetone and stirred with 1 cc. of 2 per cent hydrochloric acid in acetone. A total of about 25 gm. of recrystallized monoacetone galactose (with m.p. 157°) was obtained.

Trimethylmonoacetone-d-Galactose—5 gm. of galactose mono-

acetone, admixed with 15 cc. of acetone, were methylated with 45 cc. of dimethyl sulfate and 100 cc. of 33 per cent sodium hydroxide at 55–70°. The reaction product was extracted with chloroform, the solution dried with anhydrous sodium sulfate, and the solvent removed under reduced pressure. Trimethylmonoacetone galactose was obtained as a colorless, fairly mobile liquid, distilling at 105°, $p = 0.3$ mm. It does not reduce Fehling's solution.

Its optical rotation was

$$[\alpha]_D^{20} = \frac{-1.35^\circ \times 100}{1 \times 4.306} = -31.4^\circ \text{ (in methyl alcohol)}$$

The substance had the following composition.

4.606 mg. substance: 9.240 mg. CO₂ and 3.435 mg. H₂O.

4.900 " " : 13.095 " AgI.

C₁₂H₂₂O₆. Calculated. C 54.96, H 8.46, OMe 35.5

Found. " 54.74, " 8.34, " 35.2

Trimethyl-d-Galactose—5 gm. of trimethylmonoacetone galactose were heated at 70° for 2 hours with 50 cc. of 0.2 N sulfuric acid. The acid was neutralized with barium carbonate, the solution was filtered and concentrated under reduced pressure to a syrup which was taken up in ether and dried with anhydrous sodium sulfate, and the solvent was removed. No attempt was made to distil the free syrupy sugar. It was heated for several hours at about 80° under 0.1 mm. pressure. Yield 4.5 gm. It reduced boiling Fehling's solution. Its optical rotation was

$$[\alpha]_D^{20} = \frac{-0.18^\circ \times 100}{1 \times 4.16} = -4.3^\circ \text{ (in methyl alcohol)}$$

It had the following composition.

5.342 mg. substance: 9.500 mg. CO₂ and 3.960 mg. H₂O.

5.306 " " : 17.030 " AgI.

C₈H₁₆O₆. Calculated. C 48.75, H 8.11, OMe 41.9

Found. " 48.49, " 8.29, " 42.3

Trimethyl-δ-Galactonolactone—3.2 gm. of trimethylmonoacetone galactose were heated at 70° with 25 cc. of 0.05 N hydrobromic acid for 2 hours. The reaction mixture was transferred to a distilling flask with an equal volume of water and the liquid concentrated to its original volume to remove the liberated acetone. Bromine was

now added in portions of 0.5 gm. (total 3.5 gm.) during 4 days, the flask being kept at 35–40°. After removal of the bromine by aeration, the hydrobromic acid was removed with silver oxide and the filtered solution was titrated exactly with dilute hydrochloric acid. The filtered solution was concentrated to a syrup and heated for 4 hours at 100° (0.1 mm.) to complete the lactonization.

It had the following composition.

4.601 mg. substance: 8.245 mg. CO₂ and 2.900 mg. H₂O.

7.192 “ “ : 22.965 “ AgI.

C₉H₁₀O₆. Calculated. C 49.06, H 7.28, OMe 42.30

Found. “ 48.86, “ 7.05, “ 42.14

0.1036 gm. of lactone required for neutralization 4.71 cc. of 0.1 N NaOH (phenolphthalein as indicator). Calculated 4.71 cc. of 0.1 N NaOH.

The initial optical rotation of the lactone in water was

$$[\alpha]_D^{25} = \frac{+1.15^\circ \times 100}{1 \times 2.456} + 46.8^\circ$$

Sodium Salt—0.1036 gm. of the lactone was dissolved in 4.9 cc. of 0.1 N NaOH and made to 5 cc. with water. The rotation is calculated on the sodium salt.

$$[\alpha]_D^{25} = \frac{+0.72^\circ \times 100}{1 \times 2.45} + 29.4^\circ$$

Free Acid—0.2009 gm. of the lactone was allowed to stand for several hours with 4.8 cc. of 0.2 N NaOH and then neutralized with 4.8 cc. of 0.2 N HCl and made to a volume of 10 cc. The readings as taken in a 2 dm. tube are given in the following tabulation.

	α_D^{25}	$[\alpha]_D^{25}$
Initial.....	+0.10	2.5
60 min.....	+0.20	5.0
2 hrs.....	+0.25	6.3
5 “.....	+0.35	8.8
6 “.....	+0.40	10.0 (20 per cent lactone)
24 “.....	+0.65	16.2
48 “.....	+0.75	18.7
72 “.....	+0.80	20.0 (40 per cent lactone) constant

Addendum—When the procedure as outlined for the preparation of diacetone galactose (p. 259) is applied to glucose, the yield of diacetone glucose is close to 70 per cent of the theoretical. In addition, the whole procedure (including shaking) only requires 24 hours, thus representing a considerable saving of time as compared with previous methods.

THE CHANGES IN THE TOTAL FATTY ACIDS, PHOS- - PHOLIPID FATTY ACIDS, AND CHOLESTEROL OF THE BLOOD DURING THE LAC- TATION CYCLE

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(Received for publication, May 4, 1931)

In 1913, Mayer and Schaeffer (1) showed that the fatty acids, cholesterol, and lipid phosphorus tend to vary together in the blood plasma of various species. This observation was followed by the work of Terroine (2) and of Bloor (3) with dogs showing that in the postabsorptive state the values for total fatty acids and cholesterol in the plasma remain fairly constant for the same individual. Recently Porcher and Maynard (4) have reported similar observations with cows on full feed. Over the short period studied, the fatty acids and the unsaponifiable matter of the plasma exhibited only small variations for the same animal, from day to day or at different hours in the same day. The occurrence of constant values in spite of the periodic ingestion of food was attributed to the low fat content of the ration and to the fact that digestion and absorption in the ruminant is a fairly continuous process. Maynard and McCay (5) continued these studies with lactating cows and goats and found that when the animals were changed from a ration of normal fat content to one of low fat content there occurred a gradual drop in milk secretion accompanied by parallel drops in the blood lipids.

The foregoing observations, particularly the one showing changes in the level of blood lipids during lactation, caused us to study the plasma lipids of the cow prior to and during the period of lactation. The total lipids were determined by the oxidative method of Bloor (6). The fatty acids were obtained by difference after the determination of the cholesterol by the Liebermann-Burchard reaction, following Bloor's procedure (6). The lipid

phosphorus was determined by the method of Denigé (7) as modified by McCay (8). For purposes of comparison with the other values, it was calculated to phospholipid fatty acid by use of the factor 18.26. This factor, recently employed by Channon and Collinson (9) in their studies of the lipids in ox blood, represents the ratio of fatty acids to phosphorus in a stearyl-oleyl-lecithin.

Four Holstein cows were used in the main portion of this work. The blood was drawn from the jugular vein. The sampling, carried out at approximately the same hour each day, was begun

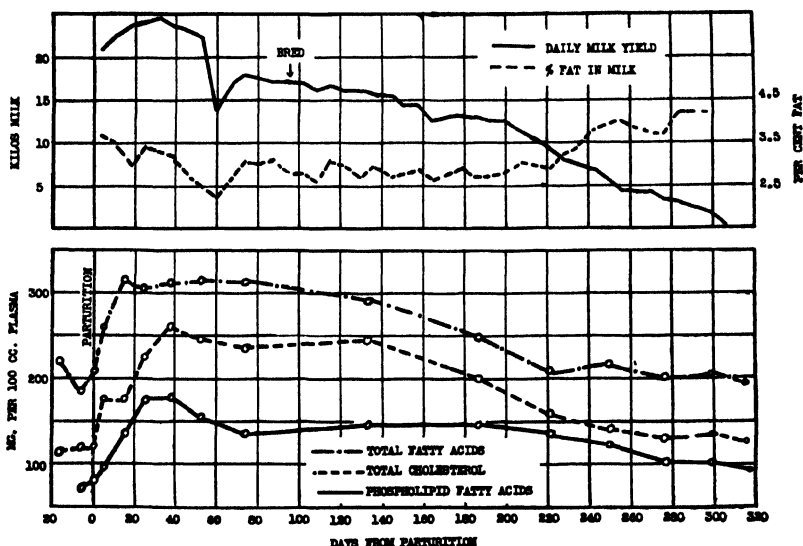


CHART 1. Lipids in blood plasma before parturition and during lactation. Milk yield and fat content data for Cow W.

when the cows were dry or nearly so and due to calve within a short time. The samples were taken at intervals of 5 to 10 days prior to parturition and for a short period thereafter, followed by sampling at less frequent intervals to approximately the 300th day after parturition. For three of the four cows the period of observation substantially covered the cycle from one dry period to the next.

Records were kept of the milk yield, its fat content, and of the feed consumed. The ration consisted of mixed hay, silage, and a grain mixture. The amounts fed were based upon the weight and pro-

duction of the animal in accordance with the Morrison standard. On a dry matter basis, the ration contained approximately 3 per cent of fat, determined as ether extract.

Results

The data obtained with the four cows are presented in Charts 1 to 4. For every animal it is seen that all of the blood lipids rose rapidly following parturition and then gradually declined. In the

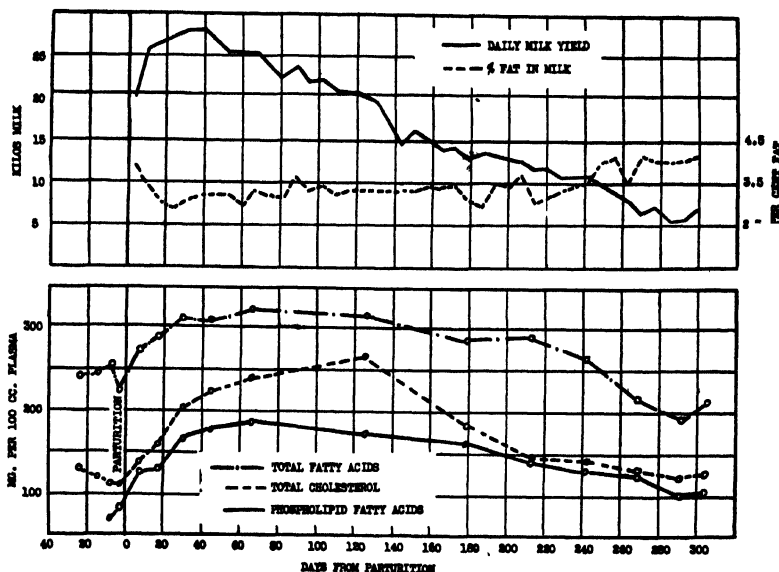


CHART 2. Lipids in blood plasma before parturition and during lactation. Milk yield and fat content data for Cow C.

case of Cow W (Chart 1) which, as shown by the curve for milk yield, had completed lactation prior to the last blood sampling, all of the lipids gradually dropped to substantially their original levels of the previous dry period. A similar course is shown for Cow C (Chart 2) and Cow L (Chart 3) which were nearly dry at the close of the experiment. A somewhat different picture is seen in the case of Cow P (Chart 4) which was still producing at a liberal rate. Here the blood values at the end remain markedly higher than the minimum levels prior to parturition.

The striking feature of these blood data is that over the cycle studied, where very large changes in the various lipids occur, the levels of all tend to remain parallel. Thus the same parallelism, which has previously been observed in fasting and non-lactating animals, is found to hold as the lipid values rise and fall during lactation, where an intense fat metabolism is occurring as the result of a high level of food intake and of the secretion of a large amount of fat in milk. A close metabolic relation among these various lipids seems evident.

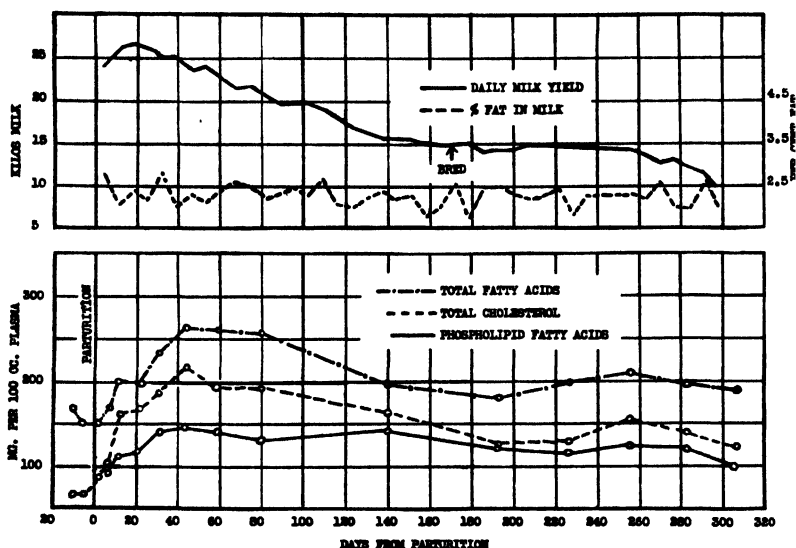


CHART 3. Lipids in blood plasma before parturition and during lactation. Milk yield and fat content data for Cow L.

The results given in Charts 1 to 4 clearly show that all of the blood lipids studied are higher during lactation than during the dry period. Meigs, Blatherwick, and Cary (10) found that the lipid phosphorus was higher during lactation, and Shope and Gowen (11) have reported a similar finding for cholesterol. Porcher and Maynard (4) also found an increase in fatty acids and unsaponifiable matter after parturition. In the present study, where the three blood lipids have been followed simultaneously throughout the lactation, we have been interested to consider any possible

relationships between their level and the course of mammary activity.

The curves for milk yield and fat percentage represent the usual lactation picture with the exception of certain variations which are explainable. The temporary, sharp drop in milk yield for Cow W (Chart 1) between the 53rd and 60th day after parturition was the result of a digestive disturbance which caused the animal to refuse a large portion of her feed. The marked drop shown for

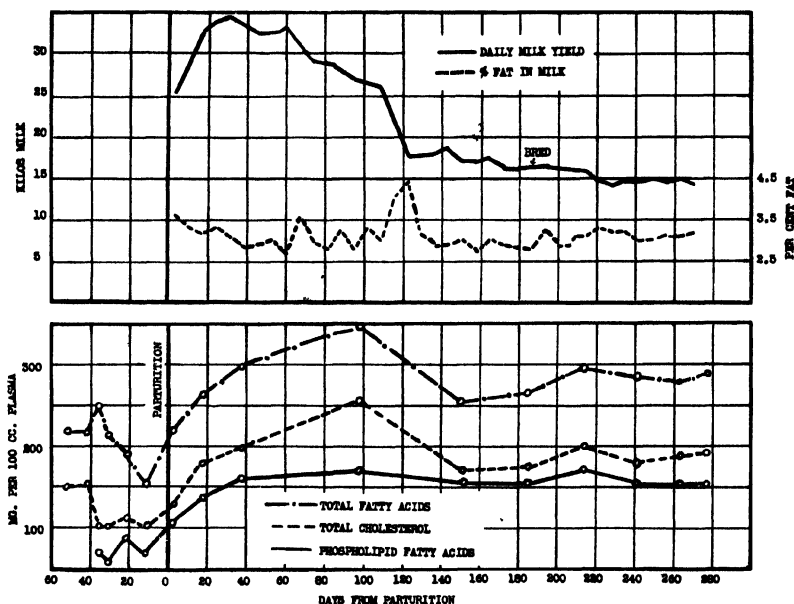


CHART 4. Lipids in blood plasma before parturition and during lactation. Milk yield and fat content data for Cow P.

Cow P (Chart 4) beginning at the 110th day was due to an acute case of mastitis. The coincident marked rise in percentage of fat is a striking but nevertheless a not unexpected result.

The general course shown for the lipids is a rapid rise after parturition followed by a gradual decline. Comparison of the curves in each chart with the corresponding one for fat content of the milk shows no relation between the level of any of the blood lipids and the percentage of fat in the secreted product. This confirms our previous observations (4, 5). The blood lipid curves do show a

relationship with the milk yield in that they rise rapidly during the first part of the lactation and then fall off gradually as lactation declines, reaching their original values as the animal enters a second dry period. The same relationship could also be said to exist with the fat yield which our data (not presented) showed to follow the yield of milk. Further evidence of this possible relationship between the plasma lipid level and lactation level is shown in the beginning of the lipid curves for Cow P. During the period covered by the first three determinations the animal was still milking. The minimum values represent the dry period.

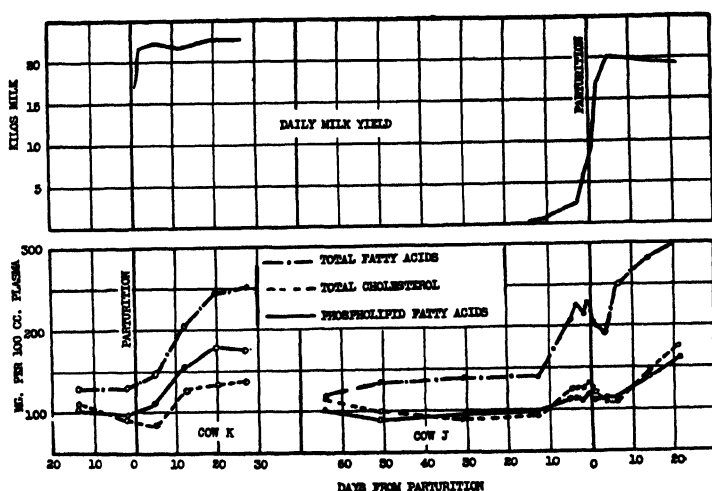


CHART 5. Changes in plasma lipids at constant food intake

In considering this possible relationship between blood lipid level and lactation level it must be remembered that these cows received food in accordance with their production and that the food and fat intakes were in general highest when the blood lipids were highest, and *vice versa*. It might be concluded therefore that the changes in blood lipids were merely the result of differences in intake and have no significant relationship to mammary activity. It seems reasonable to assume that the food level was concerned here, but we have evidence that lactation was also a factor in these blood changes.

Some indirect evidence is available from the data in Chart 4. It has been stated that this cow was still milking at the time the

first blood observations were made. During the first 25 days it was getting only 3 pounds of grain a day with the object of drying it off. After it became dry this was raised to 12 pounds, thus nearly doubling the fat intake. In spite of this increased intake, its blood lipids continued to drop to minimum values during the dry period and then rose after parturition without any increase in fat ingested. Again, when the production of this cow fell off sharply from the 110th to the 122nd day its food intake was kept at the same level for 30 days, yet during this period there were marked drops in total fatty acids and cholesterol and a slight drop in phospholipid fatty acids.

To obtain additional evidence two experiments were conducted in which the animals were held at a constant food and fat intake during the dry period and the first part of the lactation. The results are shown in Chart 5. Cow K was fed a ration which was constant in amount for 20 days prior to parturition and for 30 days thereafter, with the blood analyses as shown. A marked rise in all the blood constituents with the onset of milk secretion is evident.

Cow J was treated in a somewhat different manner. This cow was dry for 3 months prior to parturition. It was fed a constant ration throughout this period and for 3 weeks after calving, with periodic blood analyses. About 4 weeks before calving, however, an attempt was begun to initiate the secretion of milk by hand manipulation twice a day. At about the 15th day an appreciable amount of fluid was obtained which increased rapidly in succeeding days. The fluid first obtained was bloody and similar to colostrum, as shown by its appearance and chemical analysis. Its character gradually changed in the following days. On the day prior to parturition 8 kilos of a secretion which in appearance and analysis resembled normal milk were obtained. This yield obtained prior to parturition and thereafter is shown on Chart 5.

The curves for the blood lipids show that their levels remained constant up to the time that the initiation of secretion was brought about. Then they rose as the secretion increased. While the rise is small for both phospholipids and cholesterol, the uniformity of the daily observations made at this time suggests that the picture is a true one. After parturition there is a temporary drop in all the blood values followed by a further rise as the milk secretion reaches its maximum. These experiments with Cows K and J

demonstrate that lactation has an influence upon the level of blood lipids independent of any influence which the level of fat intake exerts. They confirm our observations of the parallelism exhibited by these blood constituents.

These observations that the blood lipids increase during lactation represent a different picture from what occurs with the other precursors of the organic milk constituents. Harding and Downs (12) have reviewed literature showing blood sugar to be lower in lactating than in dry cows and indicating that the amino nitrogen decreases or remains constant. They found no increase in the levels of sugar and amino nitrogen in lactating women.

Although our data show that lactation has an influence upon blood lipid level, it must not be concluded that the fat intake has no influence. Our previous experiments (4, 5) have clearly shown that there is no immediate rise in the blood lipid level of ruminants following the ingestion of fat, as occurs in dogs. Also in an experiment carried out with a dry cow in connection with the present study, the food and fat intakes were increased by 20 per cent without any effect upon the blood lipids over a period of 2 weeks. In another experiment, however, where the food of a dry cow was increased 25 per cent above the normal requirement with a 100 per cent increase in fat intake, a gradual rise in the blood lipids occurred during a 4 weeks period followed by a gradual drop when the original ration was restored. During the period of high intake the animal became excessively fat. Whether increased food may influence the blood lipid level to the same extent during lactation when fat is not being stored cannot be stated. It is clear however, from our observations that any rise in blood lipids with increased intake is a gradual one, that the rise is not solely the direct result of increased absorption. Additional metabolic factors must be involved.

The data reported in this paper represent a general survey of the course of the blood lipids in the cow from one dry period to the next, where in three out of four cases pregnancy was superimposed upon lactation. Thus no data are furnished as to the specific effect of pregnancy. Tyler and Underhill (13) have reported that cholesterol, cholesterol esters, and lecithin increase in the blood of women beginning with the 3rd month of pregnancy and continue to increase until term, and remain high for at least 2 weeks there-

after. These investigators cite several earlier but less complete studies with dogs as well as women which also showed an increase in lipids in pregnancy. However, Baumann and Holley (14) report a progressive decrease in the cholesterol and phosphatide in the pregnant rabbit followed by a rise after parturition. Metzger, in unpublished work in this laboratory, has confirmed these observations with rabbits but found with guinea pigs that the lipids tend to remain constant during pregnancy with a slight rise just before parturition. All the cows showed their lowest values just before parturition but the period when the cows were not lactating was too short and the determinations too few to furnish any information as to the changes during pregnancy uncomplicated by lactation.

SUMMARY

The total fatty acids, phospholipid fatty acids, and cholesterol have been followed in the blood of four cows during the dry period and the succeeding lactation. Following parturition there is a rapid and approximately parallel rise in all these constituents followed by a gradual drop to the original levels as the succeeding dry period is reached. Thus it is shown that a parallelism among the blood lipids, which is recognized to exist in fasting and non-lactating animals, is also exhibited as these values rise and fall during lactation where an intense fat metabolism is occurring. A close metabolic relationship among these blood lipids is thus suggested. In experiments with animals held at a constant level of food and fat intake during the dry period and the early weeks of lactation, the same rise in the blood lipids occurred following the initiation of milk secretion. This demonstrates that lactation has an influence upon the level of blood lipids independent of any effect which changes in the fat intake may exert.

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THE INTERRELATIONSHIP BETWEEN THE DIETARY FAT AND THE PHOSPHORUS DISTRIBUTION IN THE BLOOD OF LACTATING COWS

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In an earlier series of experiments (1) to determine the effect of a low fat diet upon the lipid constituents in the blood of lactating cows, we found both the total fatty acids and cholesterol of the blood were maintained at much lower levels during the periods of low fat intake. Since this lowering of the blood lipids was accompanied by a decrease in the secretion of both milk and fat by the cow, more factors must have been involved than if this lowering of the circulating lipids were merely a reflection of the fat absorption from the intestinal tract. In the course of these experiments the energy intake was equalized. All fat extracted from feedstuffs was replaced by thermodynamic equivalents of starch.

The literature affords ample evidence that the animal body can synthesize fats from carbohydrates. From such experiments as those of Jordan *et al.* (2) one must believe that the lactating animal can synthesize milk fat from carbohydrates. Little consideration has been given to the capacity of the animal for such syntheses, however. Milk-producing animals such as the cow and goat have been selected for their powers of secreting large quantities of milk and fat. Within the bodies of such animals must exist a balance that is determined on one side by the ability of certain organs to convert other constituents into fats and on the other by the secretory powers of the mammary gland. In case the secretory activity exceeds that of fat synthesis one might expect the level of blood lipids to approach the "element constante" of Terroine (3). Under such conditions one must expect the mammary gland to fall gradually in its production to the equilibrium that is determined by the level of blood lipids. The supply

of these blood lipids must come from both those, brought into the circulation from the intestinal tract, which originate directly from the food and from those synthesized within the body from carbohydrates or proteins and from depots.

Bloor (4) has found that the concentration of phosphatide in the blood is increased by feeding fat in the case of the dog. He has also attributed to the erythrocytes a function in the production of phosphatides (5). Meigs (6) believes that milk fat is "derived from the phosphatid of the blood plasma." Hence one might expect an interrelationship between the fat consumed in the diet, the phosphatides of the cells and plasma, and the secretion of fat and milk. In such considerations one must remember that the studies of Bloor were made with dogs. Such carnivorous animals have digestive tracts of relatively small capacity. They can be fed large amounts of fat which are absorbed into the blood stream quite rapidly. Hence the level of blood lipids is subject to considerable fluctuations and can be forced to levels unattainable in the ruminants. In our previous studies (1) no relation has been found between the blood lipids of the cow and feeding time. The blood lipid levels of individuals at various times of day was remarkably constant irrespective of time of feeding and milking (7). This is not surprising when one remembers that the body of a well fed cow contains more than a barrel of partly digested food which is subject to a slow gradual absorption.

In view of the probable importance of the phospholipids in the secretion of milk we have extended our earlier studies to include the distribution of phosphorus in the blood of cows allowed three different intakes of fat. Although twenty cows have been used to establish the relation between the fat fed and the milk secreted, we have studied the distribution of phosphorus in the blood of only six. All of these cows were in their 2nd or 3rd lactation month at the start of the experiment. The rations used were chosen with the object of providing optimum nutrition, aside from the possible effect of the removal of the fat. They consisted of alfalfa hay, beet pulp, and a grain mixture of the following formula.

25 pounds wheat bran.

42 " yellow hominy feed.

12 " ground soy beans.

10 " cottonseed meal.

8 pounds linseed meal.

1 pound steamed bonemeal.

1 " ground limestone.

1 " salt.

This mixture was called the high fat-grain mixture. A portion of it was extracted by the benzene process, and the fat removed was replaced by an isodynamic amount of starch to form the low fat-grain mixture. The high fat mixture and the extracted material were combined with an appropriate amount of starch to form a mixture, containing approximately 3 per cent of fat, referred to as the medium fat mixture. The analyses of the various feeds are shown in Table I.

The system of feeding for the cows on which data are reported in this paper is shown in Table II. The periods consisted of 5 weeks.

The cows were fed in accordance with the Morrison standard. The rations were recalculated weekly based upon data for milk

TABLE I
Composition of Various Feeds

Feed	Crude protein	Carbohydrates		Ether extract
		Fiber	N. F. E.*	
Alfalfa hay.....	13.86	30.14	40.07	1.65
Beet pulp.....	7.75	17.25	62.56	0.45
High fat-grain mixture.....	17.65	6.56	53.34	7.45
Medium fat-grain mixture.....	16.45	6.69	60.53	3.15
Low fat-grain mixture.....	15.85	6.83	63.31	0.99

* Nitrogen-free extract.

yield, fat content, and live weight obtained during the previous week.

Three blood samples were taken from the jugular vein of each cow during each of the feeding periods. The samples were always taken at 11 to 12 a. m. of the same day of the week. This time of day was selected since it was farthest removed from a given feeding period. The samples were taken during the 1st, 3rd, and last week of each period. All blood samples were taken over lithium oxalate.

In all analytical determinations we have followed the method detailed by Youngberg (8) with the slight modification described in our previous report (9). In Table II we present the mean values for the phosphorus distribution in the blood of the individual cows during each of the three periods. These means involve only

the last two blood samples and neglect the first in each period since in most cases the first sample was taken too close to the time of changing the ration to represent the new level of the blood constituents.

TABLE II

Distribution of Phosphorus in the Blood of Cows Maintained upon Diets of Varying Fat Content

Cow and breed	Approximate per cent ether-soluble in grain mixture	Period	Volume of red cells per 100 cc blood	Whole blood P	Cell P				Plasma P			
					Total	Lipid	Acid-soluble inorganic	Total acid-soluble	Total	Lipid	Acid-soluble inorganic	Total acid-soluble
				mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc
P, Guernsey	3	1	40	20 0	25 6	11 9	2 6	9 0	14 2	8 5	4 6	5 1
	7	2	38	20 7	25 4	12 6	2 8	7 7	15 1	10 1	4 0	4 5
	3	3	38	18 4	24 6	12 3	2 5	9 0	13 2	7 8	4 0	4 4
M, Jersey	7	1	38	20 5	29 7	11 1	2 2	12 1	14 6	8 1	4 2	4 8
	1	2	41	19 1	28 6	12 6	2 3	11 5	12 2	6 6	3 9	4 5
	7	3	40	21 7	27 7	12 6	2 6	12 7	15 4	9 2	4 4	4 7
A, Holstein	7	1	43 5	24 0	27 9	14 0	2 4	9 9	20 3	13 5	4 3	4 5
	1	2	46	21 2	28 6	13 2	3 0	10 1	14 7	9 0	4 3	4 8
	7	3	41	22 5	28 4	13 9	2 8	11 0	18 8	13 1	4 3	4 6
B, Holstein	7	1	43	22 3	28 3	12 6	1 9	10 0	17 4	11 4	4 8	5 3
	3	2	41	19 1	26 6	12 5	2 5	8 3	14 8	9 4	4 2	4 4
	7	3	41	20 5	26 9	11 1	2 5	9 8	16 7	10 6	4 1	4 5
E, Holstein	3	1	37	22 5	32 0	13 2	2 5	13 4	17 4	10 9	5 0	5 3
	7	2	38	23 8	31 0	13 4	3 2	12 8	19 6	12 7	5 1	5 4
	3	3	38	23 1	29 9	12 5	3 2	14 3	17 4	10 6	5 2	5 4
I, Jersey	7	1	40	25 7	31 4	14 8	2 7	12 0	21 0	15 4	5 2	5 6
	3	2	42	23 3	32 5	14 7	2 8	11 6	18 7	11 7	4 6	5 2
	7	3	41	23 6	28 2	12 7	2 6	12 0	18 7	12 7	4 4	4 6

In Fig. 1, we have plotted the complete blood data for two cows during three periods of feeding different intakes of fat. The trend of these data is typical for the other animals.

These data indicate that there is a marked drop in the plasma lipid phosphorus when the ration of a cow is changed from a level of approximately 7 per cent ether-soluble material in the grain

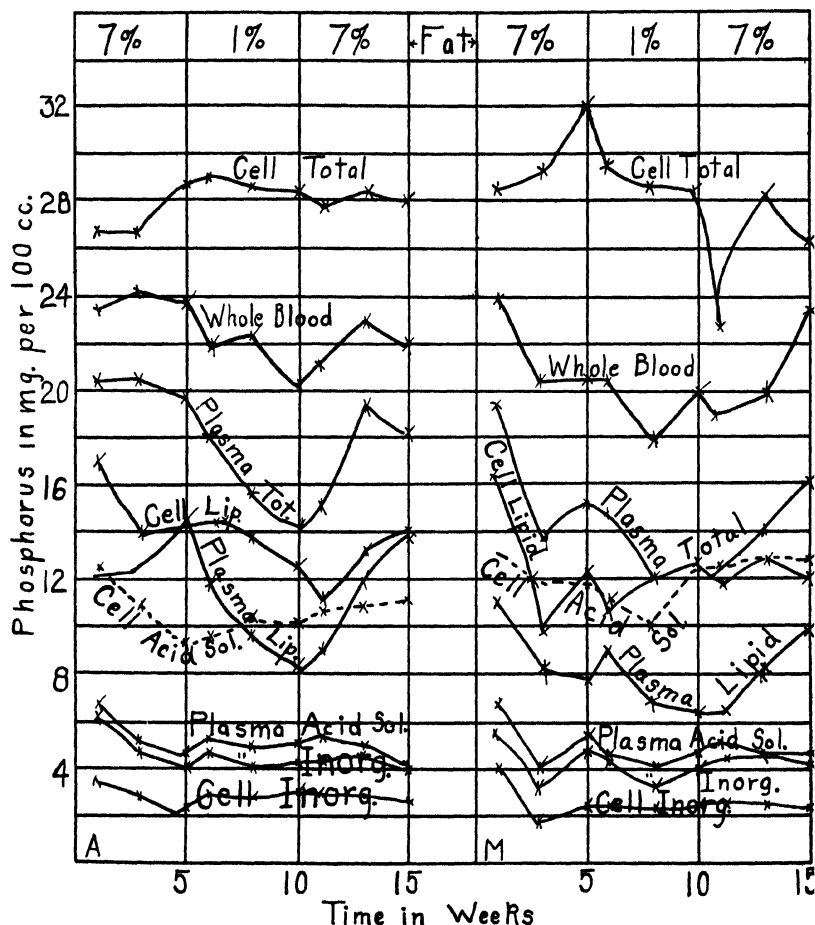


FIG. 1. The effect of dietary fat upon the distribution of phosphorus in the blood of Cows A and M.

mixture to one containing either 3 or 1 per cent. When fed the lowered intake of lipid material the blood of the lactating cow drops not only to a lower level of cholesterol, and total fatty acids as

we found previously, but also to a lower level of plasma phospholipids. This phospholipid drop is reflected in the total phosphorus

TABLE III
Average Daily Yields of Milk and Fat Secreted by Cows upon Diets Containing Approximately 1, 3, and 7 Per Cent Ether-Soluble Material in the Grain Mixture

Cow	Period	Approximate per cent ether-soluble in grain mixture	Milk secreted	Fat secreted
			kg.	kg.
A	1	7	26.5	0.87
	2	1	20.0	0.65
	3	7	18.5	0.63
Mean	1 and 3		22.5	0.75
M	1	7	12.0	0.62
	2	1	9.8	0.56
	3	7	10.4	0.62
Mean	1 and 3		11.2	0.62
E	1	3	22.0	0.66
	2	7	20.4	0.70
	3	3	18.6	0.60
Mean	1 and 3		20.3	0.63
B	1	7	30.1	1.04
	2	3	26.5	0.90
	3	7	24.8	0.91
Mean	1 and 3		27.4	0.97
P	1	3	10.9	0.46
	2	7	10.2	0.45
	3	3	8.8	0.39
Mean	1 and 3		9.8	0.43
I	1	7	19.2	1.10
	2	3	15.6	0.93
	3	7	14.8	0.92
Mean	1 and 3		17.0	1.01

of the plasma since the acid-soluble fraction remains quite constant. This change in the phospholipid of the plasma seems to

take place independently of the phospholipid of the red blood cells. This suggests that there is not a ready exchange of phospholipids between the cells and plasma in the blood of the cow or it may mean that the cells, even at the higher dietary fat levels, have a phospholipid value near that for the "element constante."

The acid-soluble fractions for both the cells and plasma have been determined. That for the plasma shows no reflection of the fat level of the diet. In certain cows there seems to be an inverse relation between the level of plasma phospholipids and the acid-soluble fraction from the erythrocytes. Such a relationship as that which appears in portions of our figures is probably fortuitous, however.

The average daily yields of milk and fat for each period are shown in Table III. Since milk secretion normally drops with advancing lactation it is not possible to make a direct comparison of yields in successive periods. However, since the decline normally occurs at a practically constant rate during the lactation months studied, it is justifiable to compare a value obtained during a given period with the average of the values obtained during periods of similar length immediately before and after. Our experiment was carried out to allow such a comparison. Thus the data for a given cow in Table III which should be compared are the values for the second period and the averages for Periods 1 and 3. On this basis it is seen that without exception the yields of milk and fat were lower during the periods of lower fat intake.

As one reviews the literature covering the distribution of phosphorus in bovine blood, one is struck by the contrast between our values and those reported by Malan *et al.* (10). The total phosphorus in the blood of the South African cattle in the area of phosphorus-deficient pastures is very low. Since no production data are given for the animals studied by Malan, one cannot determine whether this is a reflection of pasture conditions or of productive capacity.

SUMMARY

Cows have been fed for 5 week periods upon grain mixtures with approximately 1, 3, and 7 per cent ether-soluble material. These were supplemented with beet pulp and alfalfa hay. Although the calory intakes were equalized by the substitution of isodynamic

amounts of starch for the fat that was extracted, the milk and fat secreted were lower during the periods of lowered fat intake. During the period of low fat intake the phospholipids of the blood plasma and the total phosphorus of the plasma were decreased. The phospholipids of the erythrocytes were unaffected indicating that there was no compensation by the erythrocytes for the losses of the plasma. There was no appreciable change in the other phosphorus constituents of the blood. Cows that are secreting large quantities of milk and fat are unable to synthesize sufficient fat within their bodies to permit the maximum secretion by the mammary gland that takes place when a liberal amount of fat is allowed in the ration.

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FURTHER STUDIES ON CRYSTALLINE INSULIN

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A crystalline protein, having to a marked degree the specific hypoglycemic properties of insulin, was first isolated from a commercial extract of beef pancreas by Abel in 1926 (1). Since that time the workers in Abel's laboratory (2) have confirmed the earlier findings and have extended and simplified the original method of isolating the crystals. Many other investigators in different laboratories, using the Abel method, have been able to confirm their findings. Whether this crystalline substance really is insulin in its purest and most active form is a matter of the greatest interest. Perhaps the strongest confirmatory evidence of Abel's work is the work on crystalline insulin by Harington and Scott (3). These authors, using a different method of isolating the crystals, found that their crystals were identical in appearance with the crystals isolated by Abel. The results of a physiological assay on four samples of crystals, two of which were prepared by the method of Abel and two by the method of Harington and Scott, showed a uniform potency of approximately 24 units per mg. when assayed independently by four different workers. This potency value agrees well with the value obtained at the same time in the Abel laboratories (4) and with the value formerly obtained by Freudenberg and Dirscherl (5). Further, the chemical analyses of the crystals, isolated by the saponin method, agree well with the values obtained for insulin crystallized by the brucine-pyridine method.

Although the results of the chemical and physiological assay of the insulin crystals isolated from beef pancreas by different workers are now in close agreement, there are still some disturbing features in the problem of crystalline insulin. In the first place, Dingemanse (6) has reported that by adsorption of insulin on charcoal and the subsequent removal, she was able to isolate a much

more active amorphous product than crystalline insulin. This work has not been confirmed by other workers (7). Secondly, Jensen *et al.* (4) have obtained a different value for the sulfur content of crystalline insulin isolated from an active extract of hog pancreas. In a more recent communication Jensen and De Lawder (8) have confirmed this sulfur value, but were unable to obtain a uniformly crystalline preparation and point out that this difference between the sulfur value of crystalline hog and beef insulin may be due to impurities which are still present in the hog insulin. Thirdly, it has been observed by various workers that many diabetics who show localized sensitivity reactions to injections of beef insulin can be given hog insulin and show no such sensitivity reactions. Further, certain patients who show a local reaction to commercial preparations of beef insulin show similar reactions to beef crystalline insulin (9). If crystalline insulin is a chemical entity it is surprising that individuals should be hypersensitive to it—a protein substance already present in the body.

The present investigation was undertaken with the object of further clarifying some of the points at issue. It was decided to (1) isolate crystalline insulin from the pancreas of the ox, hog, sheep, and fish; (2) subject these crystals to thorough physiological and chemical analyses; (3) have the crystals, isolated from the various sources, tested on certain diabetic patients who show sensitivity reactions to the commercial preparations of beef insulin; (4) determine whether the crystalline form and physiological activity were retained after recrystallizing insulin a number of times.

The two methods at present used in isolating crystalline insulin from an active extract of beef pancreas are, (a) the Abel method, in which the crystals are separated by means of a highly buffered solution of brucine and pyridine, and (b) the Harington and Scott method, in which the crystals are separated by means of saponin or digitonin. Objections might be raised against both of these methods because of the relatively large amounts of brucine used in the Abel method, and of saponin used in the method of Harington and Scott. While the probability of having a brucine or saponin salt or adsorption compound with insulin seems rather remote because of the almost identical chemical and physiological assay of the insulin crystals isolated by these two methods, yet that possibility does exist, and further to clarify this point efforts were

made to crystallize insulin by other methods. The fact that crystalline insulin can be recrystallized with great ease from a phosphate buffer solution suggests the use of this solution as a means of isolating crystalline insulin from an extract of the ox pancreas. After a few preliminary experiments, it was found that insulin in a crystalline form could be separated with considerable ease. When this method had become more or less standardized in our hands, we attempted to isolate crystalline insulin from other sources. Great difficulty was encountered in obtaining crystals from an extract of the hog pancreas. This was also noted by the workers in Abel's laboratory (4). Even more difficult was it to obtain insulin in a crystalline form from the sheep pancreatic extract. However, by a process of many recrystallizations sufficient crystals were obtained from the various sources for a physiological and chemical assay. While the phosphate buffer method of isolating crystalline insulin cannot be recommended as the best method where a large yield of crystals is desired, in this investigation, however, it was the most desirable one.

EXPERIMENTAL

Active amorphous preparations of insulin were made from ox, sheep, hog, and fish pancreas. The method of purification was that used for the commercial preparation of beef insulin in the Connaught Laboratories. The insulin preparations had a unitage of approximately 15 units per mg. Crystallization was then accomplished by the following procedure. 60 mg. of the insulin powder were dissolved in 10 cc. of water, containing just sufficient 0.1 N HCl to dissolve the insulin. This was then added to 60 cc. of a phosphate buffer solution (pH 7), made up according to Clark (10). The solution remained clear after the addition of the insulin. The acidity was then slowly adjusted to the isoelectric point of crystalline insulin by the addition of normal ammonium hydroxide and the solution allowed to stand overnight in the refrigerator at 4°. The following morning, in the case of beef insulin, the precipitate was partially crystalline and was removed by means of a centrifuge. The amorphous protein was washed off the surface of the layer of crystals with a phosphate buffer solution (pH 6). The crystals were recrystallized by dissolving them in 5 cc. of acid water and reprecipitating at the isoelectric point from 25 cc. of the

phosphate buffer solution. A third crystallization was carried out if the resultant product was not completely crystalline. In attempting to isolate crystalline insulin from the active preparations of the hog and sheep pancreas, much difficulty was encountered. In many experiments it was necessary to reprecipitate as often as six times from a phosphate buffer solution before obtaining a product completely free from amorphous material. The fish insulin was obtained from the islet tissue of the cod, and separated in a crystalline form with comparative ease when the above method was used. The crystals from the various sources after the final crystallization were washed twice with distilled water and dried *in vacuo* for 2 days.

An experiment in which insulin was recrystallized ten times was carried out as follows: 100 mg. of ox crystalline insulin were dissolved in 15 cc. of water containing sufficient 0.1 N HCl to dissolve the insulin. This was added to 100 cc. of phosphate buffer solution (pH 7). The solution remained quite clear. Normal ammonia was added until the isoelectric point of insulin was reached. The beaker was then set in the ice-chest overnight. The following morning the crystals were examined microscopically, the supernatant liquid poured off, and the crystals removed by means of the centrifuge. They were again dissolved in 15 cc. of acid water and recrystallized from another 100 cc. of the phosphate buffer solution. This process of dissolving and recrystallizing was continued for 10 successive days. After the final crystallization, the crystals were removed by means of the centrifuge, washed twice with 5 cc. of distilled water, and dried *in vacuo* over sulfuric acid for 2 days. The weight of the crystals after ten crystallizations was 32 mg. Much of the lost insulin was subsequently recovered from the supernatant buffer solutions by allowing them to stand in the ice-chest for a few days.

Physical Properties—Microscopically the crystals isolated from active extracts of the pancreas of ox, sheep, hog, and fish were similar in appearance. Further, their physical properties, *e.g.* solubility and isoelectric point, appeared to be identical. The crystals which were recrystallized ten times retained the same general form of the other samples of crystalline insulin, but were slightly less well defined, and were not as glassy or transparent in appearance.

Physiological Assay—The physiological assays on the various samples of crystalline insulin were carried out by the mouse method. The details of this method have been described in a former communication (11). Six tests were made on each sample, forty-eight mice being used for each test. A sample of the International Insulin Standard for comparison was kindly supplied by the Insulin Committee, University of Toronto. The weights of the various samples of crystals and of the International Insulin Standard were checked by an independent worker. The various

TABLE I
Weight of Insulin for Biological Assay

	mg.
International Insulin Standard.....	7.245
Ox insulin crystals.....	2.210
Hog " "	2.780
Sheep " "	2.802
Fish " "	2.706
Beef " recrystallized ten times.....	2.845

TABLE II
Unitage of Crystalline Insulin

	units per mg.
Ox insulin crystals.....	27.2
Fish " "	27.3
Insulin recrystallized ten times.....	27.0
Hog insulin crystals.....	26.6
Sheep insulin crystals.....	25.2

samples of insulin for physiological assay were dissolved in sterile acid water (pH 2.5) containing 0.8 per cent NaCl. In making further dilutions, a similar solution was used. The weights of the various samples are shown in Table I.

Each of these samples was dissolved in 10 cc. of sterile acid water and was then given to the insulin testing department of the Connaught Laboratories for physiological assay. The results of this assay are tabulated in the protocols. When these results are calculated in terms of units of insulin per mg. of solids, the values shown in Table II are obtained.

Protocols of Mouse Assay on Various Samples of Crystalline Insulin

Source of insulin sample	Test No	International standard (5 80 units per cc)			Solution of crystalline insulin			
		Dilution	Amount injected per mouse	Convul-sion rate	Dilution	Amount injected per mouse	Convul-sion rate	Units per cc
			cc			cc		
Beef	1	1/126	0 25	3/24	1/151	0 25	4/24	7 79
	2	1/126	0 25	9/24	1/151	0 25	5/24	5 56
	3	1/101	0 25	5/24	1/126	0 25	2/24	5 35
	4	1/101	0 25	12/24	1/126	0 25	6/24	5 50
	5	1/101	0 25	7/24	1/101	0 25	10/24	6 73
	6	1/101	0 25	11/24	1/101	0 25	8/24	5 22
Mean								6 02
Hog	1	1/101	0 25	9/24	1/126	0 25	13/24	8 54
	2	1/101	0 25	19/24	1/126	0 25	16/24	6 37
	3	1/101	0 25	9/24	1/126	0 25	8/24	6 95
	4	1/101	0 25	5/24	1/126	0 25	5/24	7 24
	5	1/101	0 25	7/24	1/126	0 25	9/24	7 96
	6	1/101	0 25	12/24	1/126	0 25	12/24	7 24
Mean								7 38
Sheep	1	1/101	0 25	6/24	1/126	0 25	6/24	7 24
	2	1/101	0 25	13/24	1/126	0 25	7/24	5 57
	3	1/101	0 25	7/24	1/126	0 25	3/24	5 43
	4	1/101	0 25	11/24	1/126	0 25	13/24	7 96
	5	1/101	0 25	5/24	1/126	0 25	7/24	8 10
	6	1/101	0 25	7/24	1/126	0 25	9/24	8 10
Mean								7 07
Fish	1	1/101	0 25	10/24	1/126	0 25	7/24	6 29
	2	1 101	0 25	14/24	1/126	0 25	14/24	7 24
	3	1, 101	0 25	7/24	1/126	0 25	13/24	9 55
	4	1/101	0 25	4/24	1/126	0 25	2/24	5 79
	5	1/101	0 25	4/24	1/126	0 25	7/24	8 83
	6	1/101	0 25	10/24	1/126	0 25	8/24	6 65
Mean								7 39
Insulin recrystallized ten times	1	1/101	0 25	10/24	1/126	0 25	9/24	6 95
	2	1/101	0 25	12/24	1/126	0 25	14/24	7.96
	3	1/101	0 25	9/24	1/126	0 25	12/24	8 25
	4	1/101	0 25	4/24	1/126	0 25	5/24	7 96
	5	1/101	0 25	8/24	1/126	0 25	9/24	7 67
	6	1/101	0 25	7/24	1/126	0 25	7/24	7 24
Mean								7 67

Chemical Analyses—Samples of ox, hog, sheep, and fish crystalline insulin were sent to the Research Service Laboratories, New York, for chemical analyses. The results of their analyses showed fair agreement for carbon, hydrogen, and nitrogen with former values reported for crystalline insulin. The sulfur values, however, were not in good agreement with each other and were somewhat lower than the values formerly reported for crystalline insulin. Dr. Wintersteiner has kindly reestimated the sulfur of these crystals and has obtained the values shown in Table III. The various preparations were dried at 105° *in vacuo* to constant weight before the above estimations were made.

TABLE III
Sulfur Analyses

	per cent
Beef insulin crystals.....	3.38
Sheep " "	3.29
Fish " "	3.31
Insulin recrystallized ten times.....	3.13
Hog insulin crystals.....	3.06
" " " reestimated.....	3.26

DISCUSSION AND CONCLUSIONS

The insulin crystals, isolated from active extracts of the pancreas of ox, sheep, hog, and fish, had a similar microscopic appearance. Their solubility and isoelectric points appeared to be identical. The crystals which were recrystallized ten times retained the same general form of the original crystals.

In carrying out the physiological assay on the five samples of crystalline insulin, 288 mice were used for the assay of each sample, or 1440 mice for the five assays. It will be seen from the results obtained that there is remarkable agreement in the unitage per mg. of solids for the crystals from all sources. Four of the assays differ by less than 3 per cent. The unitage of the sheep crystals is somewhat lower than that for the other crystals. However, examination of the protocols shows that four out of the six tests gave values above the mean value for this assay. There is thus little doubt that the insulin crystals from the various sources have the same unitage. The results obtained are from 10 to 15 per cent higher than the more recent values obtained for crystalline insulin

isolated by the Abel method, or by the method of Harington and Scott. This may be explained in many ways. In the first place it may be doubted whether insulin can be assayed physiologically within this range of experimental error. However, the results of this assay and the former results published on this method of assay in Culhane, Marks, Scott, and Trevan (11) do not show any such range of error. A second possibility is that the crystals isolated by means of a phosphate buffer substance are slightly purer. A third possibility is that the control International Insulin Standard has lost potency. The standard was kept in a weighing bottle in a desiccator over phosphorus pentoxide.

The results of the chemical analyses for carbon, hydrogen, and nitrogen on the various samples of crystalline insulin were in agreement with former values which have been reported. The sulfur values of fish, ox, hog, and sheep crystalline insulin show a very close agreement with each other and are slightly higher than the former values which have been reported.

It is hoped that the results of the clinical investigation on the crystalline insulin obtained from the various sources will be published at an early date.

I acknowledge with thanks the fish islets which were supplied by Dr. A. H. Lein. I also wish to record my gratitude to Dr. Oskar Wintersteiner for making the various sulfur estimations.

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ACTION OF ACID ALCOHOL ON INSULIN

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Since the first isolation of insulin in a crystalline form (1), it has been a matter of the greatest interest to know whether this crystalline protein really represents insulin in its purest and most active form. The uniformity of the crystals, prepared in different laboratories, would, in appearance, chemical analysis, and physiological assay, point towards crystalline insulin being a chemical entity. Whether the activity of the insulin is associated with the whole protein molecule or is associated with certain prosthetic groups embedded in the protein complex is a problem for further investigation. In approaching this problem, however, one is faced with certain difficulties. When the hydrolysis of crystalline insulin is attempted with dilute acid or alkali, the physiological activity disappears long before any breaking up of the protein into peptones or amino acids can be detected. Similarly, in the hydrolysis of crystalline insulin by pepsin or trypsin, the disappearance of the physiological activity proceeds much more rapidly than the destruction of the protein (2). As the different methods of hydrolysis have led to almost negative results, an alternative and more indirect means of approach must be employed, namely to determine what group or groupings in crystalline insulin are responsible for the physiological activity. This can be done by treating the crystalline insulin with certain chemical reagents which are known to act specifically on certain chemical groups.

In this investigation we have studied the action of acid alcohol on crystalline insulin. Carr *et al.* (3) have shown that under certain conditions commercial insulin can be completely inactivated by acid ethyl alcohol; also, that under very definite conditions, this inactive insulin can be completely reactivated by means of dilute sodium hydroxide. These workers found that the inactiva-

tion could be carried out with different alcohols and acids, and that the resultant product could be reactivated. Evidence was advanced that the inactivation was due to the esterification of the carboxyl groups in insulin. The action of acid alcohol on insulin is of great practical, as well as scientific, importance since practically all commercial preparations of insulin are made from an acid alcoholic extract of the pancreas.

While the hypothesis that the action of acid ethyl alcohol on insulin is esterification would appear to be the most likely one, other possibilities exist, such as the formation of an alkyl-nitrogen group, the opening or closing of a lactone or lactam ring, an intermolecular rearrangement, or a dehydration product similar to that demonstrated by the action of alcohol on oxidized glutathione (4).

EXPERIMENTAL

In carrying out the physiological assays on the various samples of insulin in this investigation, the mouse method, as described by one of us (D. A. S.), was used (5). A standard insulin was kindly supplied us by the Insulin Committee, University of Toronto. This standard contained 20 units per cc. 1 cc. of this insulin was diluted to 400 cc. with sterile acid water (pH 2.5) containing 0.8 per cent sodium chloride. This standard was used in all the physiological assays in this investigation. In making up the dilutions of the samples of insulin of unknown strength, sterile acid water (pH 2.5) containing 0.8 per cent sodium chloride was used. Standard sterile glassware was regularly used for making the various dilutions. The ethyl and methyl alcohols were free from aldehydes.

Experiments with Acid Ethyl Alcohol—Our preliminary experiments were carried out to study the action of acid ethyl alcohol on amorphous insulin in an attempt to repeat the work of Carr. We arbitrarily chose to work with 80 per cent ethyl alcohol. Samples of 10 mg. of insulin powder (14 units per mg.) were placed in each of a series of 15 cc. centrifuge tubes. The insulin in the various tubes was dissolved in 3 cc. of 80 per cent ethyl alcohol, which had been acidified to varying degrees with hydrochloric acid (from 0.5 N to 2 N HCl). The tubes were placed in a water bath at 20° and allowed to stand at this temperature for 17 hours. Samples were then removed and the potency tested. It was found that, in some

tubes, complete inactivation had occurred. In the tube which contained 0.75 N HCl, 80 per cent acid alcohol, approximately 90 per cent of the insulin was inactivated. We considered this condition to be the most suitable for the inactivation of the amorphous insulin, since excessively drastic treatment might lead to difficulty when attempts were made to reactivate the latent insulin. Experiments were then carried out in an attempt to recover the latent potency. A series of 10 mg. samples of insulin were inactivated with 3 cc. of 0.75 N HCl, 80 per cent ethyl alcohol, by allowing them to stand for 17 hours at 20°. To each of these samples were then added 2 cc. of absolute ethyl alcohol, and immediately the protein was precipitated by addition of 10 cc. of chilled anhydrous ether. The tubes were placed in an ice bath for 2 hours. The protein which had separated was removed by means of the centrifuge, and the residue washed with 2 cc. of ether and again centrifuged. By this means we were able to precipitate the proteins quantitatively. Carr *et al.* precipitated the inactive insulin from the acid alcohol solution by the addition of acetone. In this respect we deviated from the procedure used by these workers. We were unable quantitatively to precipitate the insulin from an acid alcohol solution by means of acetone. Particularly was this true in later experiments where crystalline insulin was used. Each sample of the precipitated insulin was then dissolved in 5 cc. of water and adjusted with 0.085 N NaOH just to the alkaline side of the isoelectric point. The tubes were then chilled in an ice bath, and varying amounts of alkali added to each (from 0.5 to 2 cc. of 0.085 N NaOH). Each tube was made up to a volume of 8 cc. and allowed to stand in the ice bath for 17 hours. Samples were then removed and tested for potency. The best condition for activating the latent insulin was found to be in the tube which contained 1.5 cc. of 0.085 N NaOH. In this experiment, however, we were able to recover only 50 per cent of the activity.

Inactivation of Crystalline Insulin with Acid Ethyl Alcohol—In order to ascertain the best conditions for the inactivation and subsequent reactivation of crystalline insulin a set of experiments was carried out in a manner similar to those experiments in which amorphous insulin was used. It was found that in order to produce 90 per cent inactivation of crystalline insulin, a slightly stronger acid was necessary. The experiment which gave the

greatest activation of insulin was carried out in the following manner: 10 mg. of crystalline insulin were dissolved in 3 cc. of 1.2 N HCl, 80 per cent ethyl alcohol, and allowed to stand for 17 hours at 20°. This produced 90 per cent inactivation. The protein was precipitated by adding 2 cc. of absolute alcohol and 10 cc. of anhydrous ether. After chilling for 2 hours the protein was centrifuged off, washed with ether, and allowed to drain. The inactive insulin was then dissolved in 5 cc. of water and neutralized with 0.085 N NaOH until the protein just dissolved on the alkaline side of the isoelectric point. The tube was then chilled in an ice bath, and 2 cc. of 0.085 N NaOH were added. The volume was made up to 8 cc., and the solution allowed to stand in an ice bath for 17 hours. A physiological assay then showed that 50 per cent of the insulin had been reactivated.

The results of these experiments on the inactivation of insulin with 80 per cent ethyl alcohol and on the subsequent reactivation with alkali were somewhat disappointing, as we were unable to recover more than 50 per cent of the original activity. This may be due either to the acid concentration in the inactivating solution being too high, or to the amount of alkali used for reactivating the insulin. Control experiments on the action of alkali on insulin kept under the conditions used for the reactivation of the latent insulin showed no loss in potency. This suggested that a part of the insulin had become permanently inactivated by the acid alcohol. If the inactivation be caused by the formation of an ester, it might be anticipated that by using a higher concentration of alcohol, esterification would take place in a less acid solution. It was considered probable, also, that the methyl ester would form more readily and be more easily hydrolyzed than an ethylester of insulin. The use of methyl alcohol would have an added advantage, namely that the methyl groups attached to nitrogen, as well as the methoxy groups, could be estimated. For these reasons methyl alcohol was used in the subsequent experiments.

Action of Methyl Alcohol on Crystalline Insulin—Numerous experiments were carried out on 10 mg. samples of crystalline insulin to ascertain the best conditions for inactivation with acid methyl alcohol and the subsequent conditions for reactivation with alkali. Only the experiment which we found to be the most satisfactory is recorded in detail. 10 mg. of crystalline insulin were

weighed into a 15 cc. centrifuge tube. To this was added 0.5 cc. of 0.093 N HCl, 66 per cent methyl alcohol. When the insulin had dissolved, 4.5 cc. of 0.103 N HCl, 99 per cent methyl alcohol, were added. This gave a final solution of 0.102 N HCl, 96 per cent methyl alcohol. The tube was then placed in a water bath at 20° for 16 hours. A physiological assay at this stage showed that 92 per cent of the activity had disappeared. The inactive insulin was then precipitated by the addition of 10 cc. of cold anhydrous ether. The mixture was chilled in an ice bath for 2 hours. The protein was removed by means of the centrifuge. The precipitate was washed with 2 cc. of ether, again centrifuged, and allowed to drain. It was redissolved in 5 cc. of water, the reaction adjusted just to the alkaline side of the isoelectric point with 0.085 N NaOH, and chilled in an ice bath. A further 1.5 cc. of 0.085 N NaOH were added, the solution diluted to 7.5 cc., and kept in an ice bath for 12 hours. The solution was then diluted with isotonic saline and assayed physiologically. Approximately 60 per cent of the original activity was recovered.

In carrying out these experiments a slight precipitate developed when the insulin was allowed to stand in the acid alcohol for 16 hours. In one experiment this precipitate was separated and was found to contain 10 per cent of the protein used. This precipitate was reactivated with alkali and contained 16 units of insulin per mg. of protein. In other words, it behaved in a manner similar to the supernatant solution and merely indicated the formation of a compound, in the acid alcohol, less soluble than crystalline insulin.

In experiments which were carried out in attempts to reactivate the insulin with alkali, it was found that the amount of insulin reclaimed was independent of the amount of alkali used over a wide range of pH. Practically 60 per cent of the insulin was recovered when from 0.5 to 3.0 cc. of 0.085 N NaOH were used. Experiments were carried out in an attempt to recover the latent insulin by means of acid hydrolyses. Five solutions of equal volumes, *i.e.* 50 cc., were adjusted to varying acidities, namely pH 1.1, 1.4, 1.8, 2.4, and 2.9. To each solution were added 2 mg. of insulin inactivated with acid methyl alcohol. These solutions were heated in a water bath for 4 hours at 50°. They were then tested physiologically. Little or no potency was reclaimed. Experiments were also carried out in which the activation by means of alkali was

attempted in much more dilute solution. These experiments were unsuccessful in reactivating more than 60 per cent of the insulin. The reactivated insulin was also tested physiologically in an alkaline isotonic solution. This, however, made no difference in the amount of insulin recoverable.

The best conditions for the inactivation of crystalline insulin with acid methyl alcohol and the subsequent conditions for reactivating the insulin by means of alkali having thus been found experimentally, this work was extended to larger quantities of insulin in order to follow the methoxy and methyl-nitrogen groups during the inactivation and the subsequent reactivation of the insulin. In order to have a uniform crystalline product with which to carry out these experiments, 1 gm. of insulin crystals was recrystallized as follows: 1 gm. of insulin crystals was dissolved in 50 cc. of water, which contained just sufficient 0.1 N HCl to dissolve the crystals. This solution was then added to 1 liter of a phosphate buffer solution, pH 7. The acidity was slowly adjusted to the isoelectric point of the insulin, and the beaker allowed to stand overnight at 4°. The following morning the crystals were centrifuged off, washed twice with 10 cc. of distilled water, and dried *in vacuo* over sulfuric acid.

The methoxy and methyl-nitrogen estimations were carried out according to the micro method of Pregl (6). Using vanillin as our standard, we were able to determine the methoxy groups to within 1 per cent on duplicate analyses. As it was anticipated that some difficulty might be encountered in estimating the methoxy groups in insulin due to the presence of a very labile sulfur, analyses were made on vanillin, to which varying amounts of cystine were added. This did not interfere with the methoxy estimations. Further, the amounts of thiosulfate and cadmium sulfate in the trap were increased from 6 drops of each to 1 cc. of each. This was done in order to catch any hydrogen sulfide which might be eliminated during the methoxy estimations on insulin. The reaction bulb in the methoxy estimations was heated by immersion in a sulfuric acid bath. The bath was retained at a temperature of 140° for 20 minutes. In the methyl-nitrogen estimations the reaction bulb was heated in a bath of iron filings at 340° for 1 hour. The distilled liquid was then sucked back and the mixture heated a second time for 30 minutes at 340°. All weighings were made on a Kuhlmann

micro balance. In all instances in which insulin crystals were used the weights of the samples for analyses were always approximately the same, and nearly 30 mg. In all other estimations the samples were comparable in weight, and in the neighborhood of 20 mg.

Experiment I—500 mg. of the recrystallized insulin were dissolved in 25 cc. of 0.093 N HCl, 66 per cent methyl alcohol. To this were added 225 cc. of 0.103 N HCl, 99 per cent methyl alcohol. The solution remained clear. The flask was kept in a water bath at 20° for 16 hours. During this time a small amount of precipitate separated out. As determined in an earlier experiment, this amounted to about 10 per cent of the total protein present. The mixture was stirred, and 1 cc. was removed for a physiological assay (Sample A). A second sample (Sample B) of 75 cc. was transferred to a 250 cc. centrifuge tube. The tube was immersed in an ice bath, and 150 cc. of cold anhydrous ether were added. To the remaining 175 cc. (Sample C) were added 350 cc. of ether, and the flask was chilled in an ice bath. At the end of 2 hours both samples (Samples B and C) were centrifuged. Each sample was washed with 100 cc. of cold ether in order to remove traces of acid alcohol. After standing 1 hour they were again centrifuged. Sample B was then placed in a vacuum desiccator and dried under a low pressure (from 3 to 5 mm.) over sulfuric acid for 48 hours. The desiccator was evacuated very gradually to avoid a loss of material by spurt-ing. The dried material was used for methoxy and methyl-nitrogen estimations. Sample C was dissolved in 175 cc. of water and placed in an ice bath. When the temperature had reached 3°, chilled sodium hydroxide 0.085 N was added until the insulin just became clear on the alkaline side of its isoelectric point. The amount of alkali required was 11 cc. The solution was further diluted to 210 cc. To this solution were added 52.5 cc. of 0.085 N NaOH at 0°. The solution was kept in an ice bath for 12 hours. At the end of this time, 1 cc. of the clear solution was removed for a physiological assay (Sample D). The remaining solution was transferred to a 5 liter flask and diluted further with 20 cc. of water which had been used to wash out the original flask. The insulin was then adjusted to its isoelectric point by the addition of 5 cc. of N H₂SO₄ and completely precipitated from solution by the addition of 2 liters of alcohol and 2 liters of ether. The mixture was placed in an ice-chest for 2 days, which allowed the insulin to settle to the

bottom of the flask. At the end of this time, the clear supernatant ether alcohol solution was decanted off, and the precipitate centrifuged. The precipitate was dried under low pressure (3 to 5 mm.) for 48 hours, care being exercised during the early stages of drying. Samples of this powder were used for methoxy and methyl-nitrogen estimations. The remainder was used on experiments in which we attempted to crystallize the reactivated insulin. This was accomplished by precipitation at the isoelectric point in a phosphate buffer solution. In one experiment in which 80 mg. of the reactivated insulin were used, we were able to recover 25 mg. after its being twice crystallized. The total amount of crystalline insulin

TABLE I
Protocols of Mouse Assay on the Reactivated Recrystallized Insulin

Test No.	International standard (20 units per cc.)			Solution of crystalline insulin			
	Dilution	Amount injected per mouse	Convul- sion rate	Dilution	Amount injected per mouse	Convul- sion rate	Units per cc.
		cc.			cc.		
1	1/400	0.25	6/24	1/126	0.25	5/24	6.00
2	1/400	0.25	5/24	1/126	0.25	7/24	7.06
3	1/400	0.25	7/24	1/126	0.25	6/24	6.05
4	1/400	0.25	1/24	1/126	0.25	2/24	7.80
5	1/400	0.25	4/24	1/126	0.25	7/24	7.31
6	1/400	0.25	12/24	1/126	0.25	12/24	7.24
Mean.....							6.91

which we were able to recover from all experiments was approximately 70 mg. These crystals were assayed for potency and analyzed for methoxy and methyl-nitrogen groups. The weight of the sample for biological assay was 2.530 mg. The sample was dissolved in 10 cc. of sterile acid water.

The details and the results of the physiological assays in this experiment are as follows: 2.623 mg. of the original crystals were dissolved in 10 cc. of acid water, and 1 cc. of this solution further diluted to 126 cc. A potency test in which 288 mice were used showed a unitage of 27.1 units per mg. The inactivated insulin (Sample A) was diluted to 90 cc., and, on assaying, it was found that 91 per cent of the activity had disappeared. The reactivated in-

sulin (Sample D) was diluted to 376 cc. A potency test in which 288 mice were used showed that 57 per cent of the insulin was recovered. The results of the complete assay of the reactivated recrystallized insulin are given in Table I, and show a unitage of 27.3 units per mg. The results of the methoxy and methyl-nitrogen estimations are recorded in Table II.

We were unable to make accurate estimations for the methoxy and methyl-nitrogen groups on the amorphous reactivated insulin, due to the presence of large amounts of hydrogen sulfide. The results of the other methoxy and methyl-nitrogen estimations, as shown in Table II, indicate the formation of methyl esters and also methyl groups attached to nitrogen. The physiological activity

TABLE II
Insulin Inactivated with Acid Methyl Alcohol

	Crystalline insulin	Inactivated insulin	Reactivated insulin	Reactivated crystalline insulin
Potency, units per mg.....	27.0	2.16	15.4	27.3
Methoxy, per cent.....	0.27	3.17		0.31
	0.37	2.97		
Methyl-nitrogen (calculated as methoxy), per cent.....	1.93	5.36		1.60
	1.73	5.00		
Sulfur, per cent.....	3.31			3.60
				3.30

of the reactivated crystalline insulin was the same as that of the original crystals.

Since all or a part of the increase in the methoxy groups in Experiment I might be attributed to the adsorption of alcohol on insulin, a control experiment, in which the insulin would be immediately precipitated with ether from a slightly acid alcoholic solution, and in which no inactivation of the insulin would occur, was necessary. In this experiment the physiological activity and the methoxy and methyl-nitrogen groups were estimated.

Experiment II—150 mg. of the stock insulin crystals were dissolved in 7.5 cc. of 0.093 N HCl, 66 per cent methyl alcohol. This solution was then chilled in an ice bath, and 67.5 cc. of chilled 99 per cent methyl alcohol were added. To this solution were immediately added 150 cc. of cold anhydrous ether. The time necessary

to carry out the experiment to this stage was approximately 5 minutes. The amount of hydrochloric acid used was approximately one-tenth of the amount used in Experiment I. The precipitated mixture was allowed to stand in an ice bath for 2 hours, and then centrifuged. The precipitate was shaken with 50 cc. of cold ether and allowed to stand for another hour in the ice bath. The precipitate was again centrifuged. The residue was dried over sulfuric acid in a vacuum desiccator (from 3 to 5 mm.) for 48 hours. At the end of this time a sample of 2.408 mg. was removed and dissolved in 10 cc. 1 cc. of this solution was further diluted to 126 cc. A physiological assay in which 288 mice were used showed a potency of 26.2 units per mg. Methoxy and methyl-nitrogen estimations were carried out in duplicate on this powder. The

TABLE III
Insulin Not Inactivated with Acid Methyl Alcohol

	Crystalline insulin	Insulin precipitated from acid alcohol	Insulin recrystallized
Potency, units per mg.....	27.0	26.2	27.2
Methoxy, per cent.....	0.27 0.37	3.26 3.10	0.28
Methyl-nitrogen (calculated as methoxy), per cent.	1.93 1.73	3.48 3.56	1.8

remainder of the powder was dissolved in 10 cc. of water and added to 100 cc. of a phosphate buffer solution (pH 7). The acidity was then adjusted to the isoelectric point of the insulin. The beaker was set in the ice-chest, and the following morning the crystals were removed by means of the centrifuge. They were washed twice with distilled water and dried *in vacuo*. 1.181 mg. dissolved in 10 cc. of water, and 5 cc. of this solution further diluted to 305 cc. showed, on a physiological assay in which 288 mice were used, a unitage of 27.2 units per mg. Methoxy and methyl-nitrogen estimations were made on the recrystallized insulin. The results of these estimations are set out in Table III.

The results in Table III indicate that, when insulin is quickly precipitated by ether from a slightly acid alcoholic solution, either

the alcohol or the ether is strongly adsorbed by the insulin. The possibility of having an ester formed with the alcohol seems rather remote, first on account of the speed with which the experiment was carried out and, secondly, because of the small amount of acid used in this experiment. The possibility of having an ether or alcohol of crystallization seems a most likely one. An experiment was carried out in an attempt to crystallize this compound from an insulin alcoholic solution by the slow addition of ether. The experiment was as follows: 10 mg. of crystalline insulin were dissolved in 2 cc. of 0.093 N HCl, 66 per cent methyl alcohol, and 18 cc. of 99 per cent methyl alcohol were added. Ether was then very slowly added until the first clouding appeared. The tube was then placed in an ice-chest. The following morning, the residue which had settled out was examined microscopically. No evidence of a crystalline product was observed. The precipitate was then removed by means of a centrifuge, and anhydrous ether again added to the supernatant liquid until the first clouding of the solution appeared. The tube was again placed in the ice box. The following morning the residue was examined microscopically, but no evidence of a crystalline product could be observed.

An attempt was also made to find out whether it was the ether or the alcohol which had become adsorbed to the insulin. The following experiment was carried out: 100 mg. of crystalline insulin were precipitated from alcohol and ether, exactly as in Experiment II. After being dried for 48 hours over sulfuric acid in a vacuum desiccator (3 mm.), the insulin was dissolved in 10 cc. of water. The insulin readily went into solution, and no smell of ether could be detected. 2 cc. of this solution were removed and oxidized with a copper wire. The resorcin test for formaldehyde was distinctly positive. This result, however, may have been partially due to the presence of oxidized insulin. For this reason the remaining 8 cc. were adjusted to the isoelectric point of insulin, the tube corked and placed in an ice bath overnight. The precipitate was then removed by means of a centrifuge, and the supernatant liquid decanted off. A sample of this liquid was oxidized by means of a copper wire and the presence of formaldehyde again tested for. The test was faintly positive.

DISCUSSION

A study has been made of the action of acid alcohol on amorphous and on crystalline insulin. The experiments reported here have shown that amorphous insulin can be rendered 90 per cent inactive by treatment with acid 80 per cent ethyl alcohol. Crystalline insulin required a higher acid concentration in 80 per cent ethyl alcohol to produce the same degree of inactivation. Since we wished to follow the course of the reaction during inactivation, and as methods are known for the determination of methyl-nitrogen but not for ethyl-nitrogen linkages, acid methyl alcohol was used for the inactivation of crystalline insulin in the subsequent work. Experiments are reported for the inactivation of crystalline insulin in acid 96 per cent methyl alcohol. Many attempts were made to crystallize the inactive product. This work met with no success. It was noted in these experiments that the inactivated insulin had an isoelectric point more alkaline than the isoelectric point of crystalline insulin. This was also observed by Carr in his work with amorphous insulin. The inactivated insulin was partially reactivated by dilute sodium hydroxide. The maximum reactivation of the methylated insulin that we were able to obtain was 60 per cent. This result is rather disappointing, as Carr was able to recover quantitatively the activity of amorphous insulin inactivated with acid ethyl alcohol. The discrepancy between Carr's results and ours may have been due to the fact that in the amorphous insulin which he used, there were present protective substances which masked the action of the acid or the alkali. Or it might be that in the original insulin used by Carr there was present latent insulin which did not show activity until subsequent treatment with alkali. In our experiments, evidence was obtained to show that the sulfur had become more labile during the course of the experiment as we were unable to determine methoxy and methyl-nitrogen groups in the reactivated insulin due to the presence of large amounts of hydrogen sulfide. The reactivated insulin yielded crystals from a phosphate buffer solution which had the same microscopical appearance, isoelectric point, and physiological activity as the original insulin crystals. Further, the methoxy, methyl-nitrogen, and sulfur values were in close agreement with the former values obtained for crystalline insulin.

Judging from the analytical results which we have obtained, the nature of the action of acid methyl alcohol on crystalline insulin would appear to be 2-fold. The first action would appear to be the formation of an easily hydrolyzable alcohol-insulin compound. This compound may be an ester, but judging from the very mild conditions and the speed with which it is formed, and also the ease with which it is broken down in acid water, it would appear more feasible to consider it as an adsorption compound of alcohol with insulin, probably in the nature of an alcohol of crystallization. We were unable, however, to separate the alcohol-insulin compound in a crystalline form. The second action of the acid methyl alcohol on crystalline insulin is one in which more drastic treatment is necessary, both for the inactivation of the insulin and the subsequent treatment with alkali to reactivate the latent insulin. Analytical results show that there is an increase in the methyl-nitrogen groups during inactivation. Former results obtained on the action of nitrous acid and of formaldehyde on amorphous insulin (7) indicate that there is a free NH_2 group in insulin. The shift in the isoelectric point of the methylated insulin to the alkaline side of the isoelectric point of crystalline insulin might point towards the methyl group being attached to a NH_2 group, if one takes as an analogy the simple methyl amines which become increasingly basic on the substitution of a methyl group. On the other hand, it is rather surprising that the methyl-nitrogen linkage formed during inactivation can be broken up under such mild treatment with sodium hydroxide.

The results of the estimations of the methoxy and methyl groups attached to nitrogen are not in as good agreement with each other as was anticipated. However, the discrepancy between the results of duplicate analyses may now be partially explained. In the first place, the presence in insulin of a very labile sulfur renders difficult the estimation of the methoxy and methyl groups attached to nitrogen. In the second place, the presence of a very easily hydrolyzable insulin-alcohol compound makes estimations even more difficult. Because of the low boiling point of methyl alcohol, there is a great danger of the alcohol distilling over before reacting with the hydriodic acid. However, as these estimations were all carried out in a similar manner and with comparable weights of the substances, we feel that the values obtained for the methoxy and

methyl groups attached to nitrogen in Experiments I and II are significant, and that the inactivation of crystalline insulin in acid methyl alcohol is due to the formation of a methyl-nitrogen linkage.

SUMMARY

1. Crystalline insulin was inactivated with acid methyl alcohol and partially reactivated by means of sodium hydroxide.

2. The reactivated insulin was crystallized. The crystals had the same microscopic appearance, isoelectric point, and physiological activity as the original insulin crystals.

3. Crystalline insulin precipitated from methyl alcohol containing a small amount of hydrochloric acid yields an insulin-alcohol compound which retains the original potency and is easily decomposed by water.

4. Analytical data are presented which suggest the formation of a methyl-nitrogen compound when crystalline insulin is inactivated with acid methyl alcohol.

We wish to thank the Banting and Best Department of Medical Research for the facilities provided for carrying out the various micro analyses.

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CONTRIBUTIONS TO THE MICRO DETERMINATION OF CHOLESTEROL

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In connection with the study of the origin and the physiological significance of cholesterol in the animal body, the methods for its determination have been discussed by many investigators. The two principal methods, the Windaus gravimetric digitonin method and the colorimetric micro method of Autenrieth and Funk (1), Bloor (2), and others, have often been compared and often modified (3-6). The advantages and disadvantages of those methods are quite well known, and so far it is generally agreed that the digitonin method gives more reliable results than the colorimetric although the question may still be raised whether digitonin affects cholesterol alone or whether along with cholesterol as the digitonide are precipitated other compounds such as oxysterol (7), or other "unsaponifiable" substance (8). Recently Okey (9) published a new method for the micro determination of cholesterol in which she applied Bloor's oxidative method (10) procedure for lipid analysis, instead of weighing, to the estimation of the cholesterol digitonide prepared by the principle of Windaus.

This method interested the writer greatly, because Okey was using Bloor's oxidative method for the micro determination of phospholipid and fatty acids and it seemed desirable that all important lipids in the organism be determined by the same principle. Okey's method was repeatedly tested and it was found that when pure samples of cholesterol were used, it was recovered in good percentage although the washing of digitonide took a rather long time to remove the excess of digitonin and the results

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were not always consistent, because, as was found later, the oxidation of cholesterol digitonide needed more time than 15 or 20 minutes, the time described in the methods of Bloor and Okey. When, however, tissue extracts were used, results were not as good as when the pure cholesterol solutions were used. At least two difficulties were found. First, in the determination of free cholesterol, in which cholesterol in the alcohol-ether extract of the tissue is directly precipitated with digitonin and separated from other lipids and excess digitonin by washing with ether and hot water, the separation of digitonide from the impurities was uncertain because the alcohol-ether extract of the moist tissue apparently contained some substances besides lipids which cannot be completely removed by the treatment with ether and hot water. It so happened that in one of the tissue extracts of very low cholesterol content, the total cholesterol was less than the free cholesterol. This paradox might be explained by the probability that in the free cholesterol determination the impurities were not completely washed out and were oxidized together with digitonide.

Alkali treatment, as in the procedure for total cholesterol, seems to aid in the removal of these impurities although, as was observed by Mueller, lower values for cholesterol are obtained. Free cholesterol must, however, be determined without saponification. The asbestos mats used by Okey were also found troublesome, first because it was difficult to make mats of suitable tightness, and second because putting the whole mat into the oxidation flask seemed to be an undesirable feature, to be avoided if possible.

The second difficulty in Okey's method is in the filtration to isolate the digitonide. This must be emphasized more than the former one, since the impurity remaining in the filter after washing was in most cases small in amount and would not make a great difference in the ordinary determination. But the difficulty in the filtration of digitonide in the presence of large amounts of lipids makes the procedure tedious and uncertain. These difficulties have been largely overcome by the modifications noted below in which an attempt has been made to find the optimal conditions for oxidation also. Results by the modified micro method are compared with those by the colorimetric method.

Principles of the Modified Method

Cholesterol in the lipid extract is precipitated by adding excess of digitonin and evaporating to dryness. When hot water is added, the superfluous digitonin is readily dissolved in the water but the insoluble digitonide and the remaining lipids form a heavy colloidal liquid that can be filtered only with difficulty. When, however, acetone is added to the water the lipids are immediately dissolved in it to a considerable extent, the cloudy colloidal mixture becomes clear, and the filtration to remove the excess of digitonin and lipids from the precipitate of cholesterol digitonide can be accomplished in a few minutes, leaving digitonide on the mat of the filter. The addition of acetone in the above procedure was intended mainly to make the filtration easy, but it also removed a good deal of lipid from the mixture and rendered easier the later removal of all the lipids by the washing with ether. The digitonide is then dissolved in hot absolute alcohol in the filter tube, the alcohol being kept hot by means of a steam-heated jacket, and filtered into oxidation flasks. The alcohol is evaporated and the digitonide is oxidized by Bloor's method. *Absolute* alcohol dissolves cholesterol digitonide easily at its boiling temperature. After the filtration, the mat of the filter is very often found to be covered with a small amount of some colored substance, which emphasizes the desirability of the solution of the digitonide in alcohol and the filtration.

Procedures

For details as to the procedure the reader is referred to the communication of Okey (9).

I. Free Cholesterol—To about 20 cc. of alcohol-ether extract of the tissue (containing about 1 mg. of cholesterol) in a small flask are added 3 cc. of 0.5 per cent alcoholic digitonin solution and the mixture is heated to dryness on the steam bath. The last traces of alcohol are removed by a current of CO_2 , then 10 cc. of water are added and heated gently until the contents of the flasks begin to boil. It is kept boiling for a few minutes with constant gentle shaking or stirring with a glass rod. The excess of digitonin is completely dissolved in the hot water. Then 20 cc. of acetone are added to the flask, the mixture is shaken well and then filtered through a sintered glass filter of about 15 cc. volume. (*Note*—The

writer has been using the filter marked "4 G 4 Schott & Gen. Jena.") The filtration proceeds quickly and the inside of the flask and the filter are washed with acetone again. The contents of the flask, now almost entirely transferred to the filter, are washed with ether two or three times to remove lipids from the digitonide. Each time the ether should stay on the filter for a minute or 2, during which time the precipitate is stirred up with a glass rod, then sucked dry. The filter is removed from the filtration flask and set on the oxidation flask (glass-stoppered Pyrex Erlenmeyer); about 20 cc. of purified absolute alcohol are heated to boiling in the flask used for the precipitation of cholesterol with digitonin. With this hot alcohol the digitonide in the filter is dissolved and filtered out into the oxidation flask easily. The filter is kept warm during that procedure by the use of a steam jacket consisting of a copper coil in which the filter fits. The alcohol is evaporated and the last trace is removed by a current of CO_2 , then the digitonide is oxidized by Bloor's method.

II. Total Cholesterol—The bound cholesterol is not directly measured in this method, but its value is obtained by subtraction of free cholesterol from total cholesterol which is determined as follows: An adequate amount of the tissue extract containing about 1 mg. of cholesterol is pipetted into a 100 cc. flask and saponified with 2 cc. of sodium ethylate, as in the total lipid determination by Bloor (prepared by dissolving 2.5 gm. of metallic sodium in 100 cc. of absolute alcohol) and heated for 15 or 20 minutes on the steam bath. This time is long enough for the completion of the saponification as may be seen later. If the alcohol-ether extract of tissues does not contain much cholesterol, so that a larger amount of the extract has to be taken, the extract should be evaporated to some extent before adding the alkali, since the time of saponification shouldn't be unnecessarily long because of the danger of the modification of cholesterol by concentrated alkali at the relatively high temperature. At the end of the saponification the contents of the flask should be of small volume. Carbon dioxide is introduced into the flask, serving a double purpose: removal of excess liquid and changing the NaOH to the much less strongly alkaline sodium carbonate or bicarbonate and stopping promptly the action of the alkali on the cholesterol. The dried mass in the flask is neutralized with 1 cc. of 10 per cent sulfuric

acid and completely extracted with petroleum ether. The petroleum ether extract is evaporated to small volume, dissolved in 5 cc. of alcohol, and 3 cc. of 0.5 per cent alcoholic digitonin solution are added; then the mixture is heated to dryness on the water bath. 10 cc. of water are now added to the flask which is heated to dissolve the excess of digitonin. The further procedures to remove the excess digitonin and lipids from the precipitate of cholesterol digitonide and so on are the same as those described for the free cholesterol determination.

EXPERIMENTAL

I. Oxidation of Cholesterol by Bloor's Method, and the Determination of Free Cholesterol—On the assumption that cholesterol has

TABLE I
Influence of Time upon the Oxidation of Cholesterol and Its Digitonide

Cholesterol content of oxidation flask	Time of oxidation (at $124 \pm 2^\circ$)	Cholesterol found	Recovery
mg.	min.	mg.	per cent
1	20	0.88	88
1	30	0.96	96
1	40	0.96	96
1	60	0.89	89
2	30	1.88	94
1 (as digitonide)	20	0.87	87
1 " "	30	0.93	93

been precipitated with digitonin completely and separated from other substances, it was necessary to investigate whether or not the oxidation that follows is complete under the conditions described in Bloor's oxidative determination of lipids.

As the standard solution of cholesterol, a 0.1 per cent chloroform solution of cholesterol prepared from gall stones and having a melting point of 145° has been used. 1 cc. of this solution that contains 1 mg. of cholesterol and which would require 3.92 cc. of 0.1 N $K_2Cr_2O_7$ for oxidation is taken and heated for 20 minutes; the amount of dichromate used up was found low, about 10 per cent lower than the theoretical value as may be seen in Table I. The time of oxidation was changed from 20 to 60 minutes at temperatures of $124 \pm 2^\circ$. (See Table I.)

As may be seen in Table I, the oxidation of cholesterol by Bloor's oxidative method at a temperature between 122 and 126° seems to be almost complete when the time of oxidation is 30 minutes although the deviation is still 4 per cent, a deviation which might be allowed as an experimental error.

The oxidation of cholesterol digitonide which is also shown in Table I is also better when the time is prolonged to 30 minutes.

As controls, solutions containing 2 cc. of $N K_2Cr_2O_7$ and 5 cc. of silver dichromate were used. Absolute alcohol was used to dissolve the cholesterol digitonide. All these controls yielded blank determinations.

TABLE II
Recovery of Added Cholesterol (Oxidation at $124 \pm 2^\circ$)

Tissue extracts	Cholesterol found*		Difference from theoretical amount	Recovery
	Per 15 cc. aliquot	After adding 0.93 mg. cholesterol		
	mg.	mg.	mg.	per cent
Mouse tumor†.....	0.54	1.40	0.07	95
“ liver.....	0.44	1.34	0.03	98
Rat tumor†.....	0.25	1.22	0.04	103
“ liver.....	0.36	1.25	0.04	97

* Average of each three determinations.

† Mouse adenocarcinoma, Jensen rat sarcoma. The tissues were extracted in the proportion of about 1 gm. of moist tissue in 100 cc. of the solvent.

This method was applied for the determination of free cholesterol in the alcohol-ether extract of the tissues of mice and rats and it was found as shown in Table II that when cholesterol was added to the tissue extract, it was recovered with only a small per cent of loss, showing that the precipitation of cholesterol by digitonin and the separation and oxidation of the digitonide as above described were satisfactory.

II. Determination of Total Cholesterol—The procedure for this determination has already been described. Since free cholesterol can be exactly measured, the question in the determination of total cholesterol is concerned simply with the completeness of the

saponification of bound cholesterol and the quantitative precipitation of cholesterol digitonide that follows. As the material, cholesterol palmitate of melting point 78° synthesized from cholesterol and palmitic acid has been used. The cholesterol content in it has been calculated to be 61.7 per cent. The factor for the oxidation of cholesterol palmitate is 3.90, that is, it needs 3.90 cc. of 0.1 N $\text{K}_2\text{Cr}_2\text{O}_7$ for the oxidation of 1 mg. of cholesterol palmitate. 2 cc. of 0.1 per cent chloroform solution of cholesterol palmitate were measured into the oxidation flask and heated on the steam bath to evaporate the chloroform. The use of CO_2 helps the removal of chloroform from the flask. In another series of

TABLE III

Oxidative Determination of Bound Cholesterol, and the Influence of Alkali Treatment in Saponification on Cholesterol

Materials	Time for saponi- fication	No. of experi- ments	Cholesterol		Devia- tion
			In ali- quot*	Found	
	min.		mg.	mg.	per cent
Cholesterol palmitate.	15-20	10	1.19	1.12	-5.9
“ “	15	2	1.19	1.14	-4.2
“ “	20	2	1.19	1.14	-4.2
Free cholesterol.	20	5	0.93	0.91	-2.1

* 2 cc. of 0.1 per cent chloroform solution of cholesterol palmitate were taken as an aliquot which was found to contain 1.94 mg. of cholesterol palmitate by oxidative determination; consequently the cholesterol content in it was calculated to be 1.19 mg.

experiments 2 cc. of the same cholesterol palmitate solution were measured into an Erlenmeyer flask, the chloroform evaporated, then 20 cc. of alcohol-ether mixture and 2 cc. of sodium ethylate solution to saponify the bound cholesterol were added. The mixture was heated on the steam bath for 15 or 20 minutes and otherwise treated as described above. The results are tabulated in Table III.

It may be seen in Table III that cholesterol as an ester is determined by this method with a loss of 5.9 per cent on an average. It was feared that the alkali treatment in saponification would destroy cholesterol. The results however showed that this did not take place to any considerable extent under the conditions

described above. The saponification is complete within 15 minutes, but 20 minutes may be better for evaporation of the contents of the flask to a smaller volume which finally is brought to dryness by the current of CO_2 , since 20 minutes of heating for the saponification doesn't change the results as indicated in Table III.

TABLE IV
Comparison between Colorimetric and Microoxidative Methods in the Determination of Cholesterol in Tissue Extracts

Tissues	Cholesterol (total) content in 100 cc		Variations from colorimetric value
	Colorimetric method	Oxidative method	
	mg	mg	per cent
Mouse liver*	4.60	4.00	-15
" brain	13.70	12.70	-7.3
" spleen	3.05	2.58	-15.7
Rat liver†	2.52	2.14	-15.1
" kidney	4.03	3.64	-9.2
" skin	3.70	2.94	-20.5
" muscle	2.00	1.93	-3.5
Rat liver (a)‡	2.96	2.33	-21.7
" (b)	2.22	1.72	-22.5
" (c)	1.90	1.79	-5.8
Rat tumor (a)	3.18	3.33	+4.7
" (b)	2.59	2.32	-10.4
" (c)	2.34	2.13	-9.0

* The mice were fed on high cod liver oil diet for about 2 months and bore adenocarcinoma. Ten mice were killed and the tissues were combined for the extraction.

† These extracts were supplied by Dr. Bloor. They contain the material from about 1 gm. of moist tissue in 100 cc. of alcohol-ether mixture except the muscle extract in which about 2 gm. of the tissue were in 100 cc.

‡ The rats were fed with high cod liver oil diet in (a), high butter diet in (b), high coconut oil diet in (c) for about 5 weeks. Tumor was Jensen's rat

III Comparison between Oxidative and Colorimetric Methods for Cholesterol in Tissue Extracts—It is of interest and importance to compare these two methods. It has been believed that the digi-

tonin method of Windaus gives considerably lower results than does the colorimetric. Will the same results be obtained if the oxidative micro method is used instead of the gravimetric digitonin method? Comparison has been made on the tissue extracts, selected at random, of rats and mice fed on normal and special diets containing different lipids in the study of lipid metabolism in tumors regarding which the writer wishes to report later. The tissue extracts were prepared so that the extract from approximately 1 gm. of tissue was contained in 100 cc. of alcohol-ether mixture. The results are shown in Table IV.

Generally speaking, the cholesterol values in the tissue obtained by the colorimetric method are about 15 per cent higher than by the oxidative one. It is interesting to note in Table IV that the difference between the two values by the colorimetric and oxidative methods is not alike in all tissues. In liver the differences are in most cases between 15 and 20 per cent, in spleen about 15 per cent, in skin about 20 per cent, and in kidney, brain, tumor, and muscle the differences are under 10 per cent.

SUMMARY AND CONCLUSION

The need of a micro method for cholesterol determination other than the colorimetric one has been recognized for a long time. Since the gravimetric method, however it may be modified, demands relatively large amounts of material, a new method would have to be based upon a different principle. Okey applied Bloor's oxidative method of lipid determination to the cholesterol digitonide and her method seemed to fill the need but in practice it had at least two disadvantages. By the modifications described in this communication, those difficulties have been largely, if not completely, removed. The oxidation of cholesterol or cholesterol digitonide, however, is not as easy as is the determination of phospholipid and fatty acid and the conditions of oxidation of cholesterol have therefore been modified. The modifications of Okey's procedure may be summarized as follows: (a) By the use of acetone the separation of cholesterol digitonide from the excess of digitonin and lipids is greatly facilitated. (b) By solution of the digitonide in hot absolute alcohol and filtration it can be entirely freed from impurities and delivered in a form convenient for further treatment. The whole procedure is considerably

simplified and the modified method can be carried out easily and quickly with accurate results.

Comparative results by the colorimetric and oxidative methods on tissue extracts show that the colorimetric method gives about 15 per cent higher values than the oxidative method and that the difference is not the same for all tissues.

The writer wishes to express his indebtedness to Professor W. R. Bloor under whose direction this work has been done.

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STROPHANTHIN

XXII. THE CORRELATION OF STROPHANTHIDIN AND PERIPLOGENIN WITH DIGITOXIGENIN AND GITOXIGENIN

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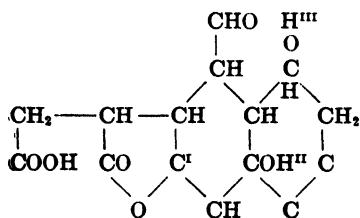
(Received for publication, May 23, 1931)

In a recent communication¹ the structural relationship between strophanthidin and periplogenin has been described. This correlation was made possible by the conversion of α -isostrophanthidic acid (partial formula I) into its desoxo derivative (II), which was in turn found to be identical with isoperiplogenic acid. This identity was confirmed by a comparison of the methyl esters obtained from both sources as well as by the comparison of the oxidation products of these esters in which the secondary hydroxyl group has been oxidized to the ketone, isoperiplogonic methyl ester (IV). Our experience therefore in regard to transformations with certain derivatives of isostrophanthidin at once gave promise of becoming applicable to analogous derivatives of isoperiplogenin. α -Isostrophanthonic dimethyl ester (III) differs from isoperiplogonic methyl ester (IV) by its possession of a carbomethoxyl group in place of the methyl group of the latter compound. In previous work² it has been shown that since OH^{II} is in a position β to the carbonyl group, isostrophanthonic dimethyl ester may be readily converted into the unsaturated anhydroisostrophanthonic dimethyl ester. Isoperiplogonic methyl ester has now been found to yield very readily the analogous unsaturated *anhydroisoperiplogonic methyl ester* (V). And just as the former unsaturated compound on hydrogenation with palladium and hydrogen was converted into a mixture of stereoisomeric saturated desoxy- α -

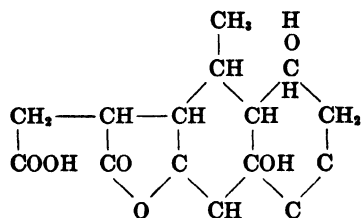
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² Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 813 (1927).

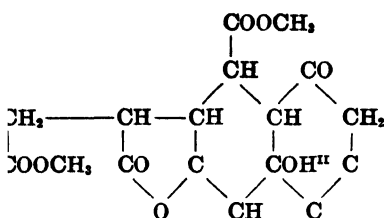
isostrophanthonic dimethyl esters, anhydroisoperiplogonic methyl ester was found to behave similarly. The latter on hydrogenation yielded a mixture of saturated *desoxyisoperiplogonic methyl esters* (VI) from which two isomers have been isolated. One of these was more readily separated because of its sparing solubility. It melted at 251°. The second isomer which appeared to have been formed in smaller amount was separated from other more soluble hydrogenation products. After repeated recrystallization from ether it was obtained in a pure form which melted at 192–193.5°.



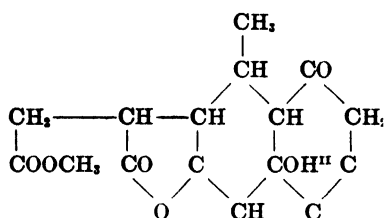
I



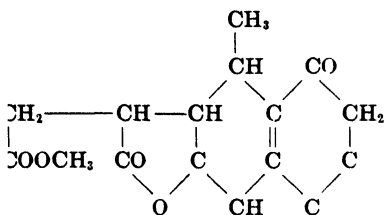
II



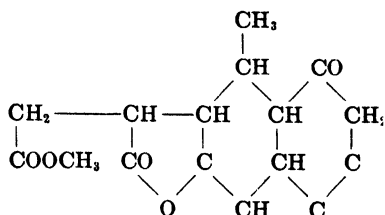
III



IV



V



VI

In previous work on digitoxigenin reported from this laboratory³ its conversion into isodigitoxigenin was described and the rela-

³ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **78**, 573 (1928).

tionship of this iso derivative to its parent aglucone was shown to be the same as that which had already been established between isostrophanthidin and strophanthidin. In the course of this work isodigitoxigenin on saponification of its lactone group was converted into the lactol acid, isodigitoxigeninic acid. The latter as the methyl ester was oxidized by chromic acid to the lactone ester, isodigitoxigonic methyl ester, in which the secondary acylatable hydroxyl group was simultaneously oxidized to carbonyl. This ~~l~~ keto lactone ester has the same formula, $C_{23}H_{34}O_6$, as the above desoxyisoperiplogonic methyl ester obtained from periplogenin. Since the melting point of the digitoxigenin derivative was practically the same as that observed in the case of the more soluble of the two desoxyisoperiplogonic methyl esters it was of interest to make a careful direct comparison of the substances obtained from both sources. We believe that this comparison has definitely established the identity of the isodigitoxigonic methyl ester and the more soluble desoxyisoperiplogonic methyl ester. This was shown by melting points, mixed melting points, crystalline form, rotation, and general properties. Further, on saponification, the periplogenin derivative yielded a *desoxyisoperiplogonic acid* which could not be distinguished from isodigitoxigonic acid, as regards melting point, crystalline form, and other properties.

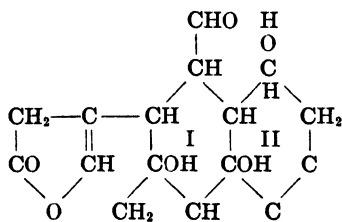
For additional confirmation of the identity of the substances from both sources a comparison was made of the substances obtained from each by isomerization with hydrochloric acid. Isodigitoxigonic acid was converted by this reagent into γ -isodigitoxigonic acid which was characterized further by its *methyl ester*. Desoxyisoperiplogonic acid of strophanthidin origin yielded similarly a γ -acid and *methyl ester* which agreed in properties with the above γ -derivatives from digitoxigenin.

From these results periplogenin is hydroxydigitoxigenin in which the extra hydroxyl group is OH^I of the strophanthidin and periplogenin molecules. The correlation of strophanthidin, periplogenin, and digitoxigenin appears, therefore, to be definitely established. Since gitoxigenin has been shown to be hydroxydigitoxigenin,⁴ the isomerism of gitoxigenin and periplogenin both of the formula, $C_{23}H_{34}O_6$, consists solely in the position of this extra

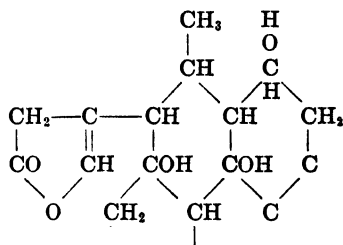
⁴ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **86**, 199 (1930).

hydroxyl. In periplogenin it is of tertiary and in gitoxigenin of secondary nature.

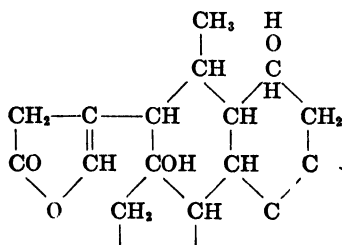
In the work of Windaus, Westphal, and Stein⁵ hexahydrodigitigenin has been shown to yield a ketone which on further oxidation gives rise to a dibasic acid with ring cleavage. On distillation this acid gives a "Brenzketon," thus establishing the fact that the secondary hydroxyl group is attached to a carbon atom of a 6- or possibly a 7-membered ring. The results will be published shortly of a study of ring II of strophanthidin, which definitely restrict its size to a 6-membered ring. The relationships between the four aglucones—strophanthidin, periplogenin, digitoxigenin, and gitoxigenin—are therefore given in the following partial formulæ.



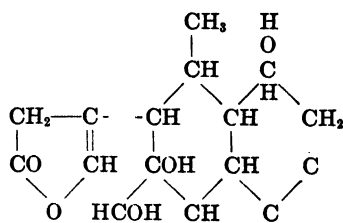
Strophanthidin



Periplogenin



Digitoxigenin



Gitoxigenin

In these formulæ the unsaturated lactone side chain is for the moment arbitrarily attached to carbon atom (7) although carbon atom (5) is also a possibility. This question is at the moment under investigation. The numerical designations given to the

⁵ Windaus, A., Westphal, K., and Stein, G., *Ber. chem. Ges.*, **61**, 1854 (1928).

* Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **67**, 614 (1926).

EXPERIMENTAL

Anhydroisoperiplogonic Methyl Ester—0.4 gm. of isoperiplogonic methyl ester (of both strophanthidin and periplogenin origin) was refluxed for 18 minutes in a mixture of 30 cc. of methyl alcohol and 1.2 cc. of hydrochloric acid (1.19). The solution was diluted with water and a moderate excess of sodium acetate solution was added. The lustrous platelets which separated were collected with water. The yield was 0.35 gm. After recrystallization from methyl alcohol, the keto ester formed sparingly soluble broad needles or flat prisms which melted at 225–226° after preliminary softening.

3.962 mg. substance: 2.942 mg. H_2O , 10.430 mg. CO_2 .

$C_{24}H_{32}O_5$. Calculated. C 71.94, H 8.07

Found. " 71.80, " 8.30

Desoxyisoperiplogonic Methyl Ester (Isodigitoxigonic Methyl Ester)—0.6 gm. of anhydroisoperiplogonic methyl ester in acetic acid solution was shaken with 0.1 gm. of palladium black and hydrogen. 1 mol of H_2 was absorbed during 30 minutes. The filtered solution was concentrated to dryness under diminished pressure and the residue was dissolved in methyl alcohol. Concentration of the resulting solution yielded three crops of a rather sparingly soluble substance. When recrystallized from methyl alcohol it separated as long flat needles or plates melting at 251–252°. 0.18 gm. of pure substance was thus obtained.

$[\alpha]_D^{24} = -44^\circ$ ($c = 1.250$ in pyridine).

3.580 mg. substance: 2.820 mg. H_2O , 9.417 mg. CO_2 .

$C_{24}H_{34}O_5$. Calculated. C 71.60, H 8.52

Found. " 71.74, " 8.80

The mother liquor from the final crop of the above substance amounted to about 5 cc. On dilution with water, a voluminous precipitate formed which was collected with water. After repeated recrystallization from ether, 0.15 gm. was obtained as rectangular platelets which melted at 192–193.5°. An identical melting point was shown by isodigitoxigonic methyl ester which had been obtained from digitoxigenin. A mixture of the two substances showed no depression. The substances from both sources were again recrystallized from ether and their melting points as well as that of the mixture remained unchanged. Both substances

gave a practically colorless solution in H_2SO_4 , which very slowly changed on warming to a pale yellow with slight greenish fluorescence.

The substances of periplogenin and digitoxigenin origins gave the following rotations respectively.

$$[\alpha]_D^{25} = -40.5^\circ \text{ (c = 1.025 in alcohol).}$$

$$[\alpha]_D^{25} = -39.5^\circ \text{ (c = 0.905 in alcohol).}$$

The analysis of the substance from digitoxigenin has already been reported.³ The analytical figures obtained from the periplogenin product are as follows:

4.057 mg. substance: 3.165 mg. H_2O , 10.680 mg. CO_2 .

$\text{C}_{24}\text{H}_{34}\text{O}_8$. Calculated. C 71.60, H 8.52

Found. " 71.79, " 8.73

The same series of substances was obtained from isoperiplogonic methyl ester of strophanthidin origin.

Desoxyisoperiplogonic Acid (Isodigitoxigonic Acid)—The more soluble desoxyisoperiplogonic methyl ester was saponified by gentle warming of a suspension in dilute alcoholic solution containing a slight excess of alkali. On gentle acidification the acid separated as flat needles. After collection with water it was recrystallized by careful dilution of an alcoholic solution. It deposited as flat needles or platelets which were indistinguishable in form from isodigitoxigonic acid. It melted at $206\text{--}208^\circ$ and showed no depression when mixed with a freshly recrystallized sample of isodigitoxigonic acid. The latter melted identically and not at $212\text{--}213^\circ$ as previously reported.³ The analysis of the material from digitoxigenin has been reported; that for the periplogenin derivative was as follows:

3.910 mg. substance: 2.905 mg. H_2O , 10.220 mg. CO_2 .

$\text{C}_{23}\text{H}_{32}\text{O}_8$. Calculated. C 71.08, H 8.31

Found. " 71.28, " 8.31

γ -Isodigitoxigonic Acid (γ -Desoxyisoperiplogonic Acid)—50 mg. of isodigitoxigonic acid were treated with 1 cc. of HCl (1.19) at 20° . Solution occurred readily and after a few minutes crystals of the γ -acid appeared. After standing 45 minutes the solution was carefully diluted and the crystals were collected with water.

The same substance was also prepared by oxidation of γ -isodigitoxigenic methyl ester in acetic acid solution with Kiliani's CrO_3 solution.

Isoperiplogenic Acid from α -Isostrophanthidic Acid Semicarbazone—In more recent work it has been found desirable to modify somewhat the procedure previously given for the preparation of desoxoisostrophanthidic acid. The contents of the bomb tubes were washed into a separatory funnel with water, the solution was made acid to Congo red with hydrochloric acid and then immediately extracted with chloroform before crystallization occurred. The resin remaining after concentration of the chloroform solution was dissolved in acetone. The solution was diluted slightly and then acidified to Congo red. On standing, crystallization occurred which was facilitated by further dilution. The substance was then recrystallized by careful dilution of its acetone solution.

STROPHANTHIN

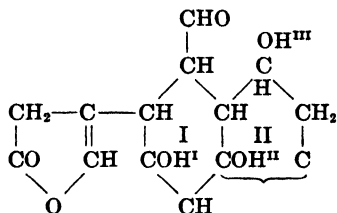
XXIII. RING II OF STROPHANTHIDIN AND OF RELATED AGLUCONES

By WALTER A. JACOBS AND EDWIN L. GUSTUS

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

(Received for publication, May 21, 1931)

In the strophanthidin molecule one of its four rings which has been designated as Ring I (Formula I) has been found to be 6-membered. This conclusion has been based upon a number of previously presented observations, especially those which have had to do with the behavior of trianhydrostrophanthidin on hydrogenation and on oxidation.¹ Adjoining Ring I is a second ring, Ring II,



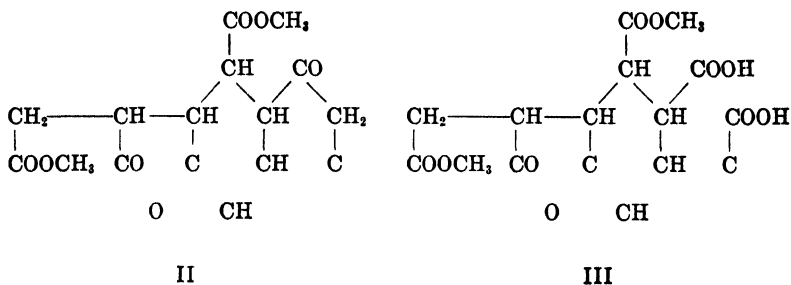
to which is attached the secondary, acylatable hydroxyl group (OH^{III}) and the determination of the size of this ring has been the subject of long inquiry by us. This we believe has now been brought to a definite conclusion.

The solution of the problem was first attempted by the application of the usual procedure of oxidizing the secondary hydroxyl group (OH^{III}) to carbonyl and then attempting ring cleavage to a dicarbonic acid which on distillation should yield either an anhydride or a cyclic ketone. Unfortunately this method was not

¹ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 805 (1927).

applicable to strophanthidin itself or its immediate derivatives because of complications caused by the proximity of the tertiary hydroxyl (OH^{II}) which is β to any resulting ketone. In addition, the secondary hydroxyl^{III} (and therefore the ketonic group) is γ to the aldehyde group (or carboxyl group produced from it) attached to Ring I.

In order to circumvent these difficulties attempts were made to accomplish this purpose with the two previously described² hydrogenation products of anhydroisostrophanthonic dimethyl ester, the isomeric desoxy- α -isostrophanthonic dimethyl esters (II) in which OH^{II} has been replaced by hydrogen and the carboxyl group has been converted into a relatively stable ester group. One of these isomers ($[\alpha] = +12$), which was formed in larger amount during hydrogenation, gave on oxidation only non-crystalline products which proved to be useless for our purpose. The isomer obtainable in smaller amount yielded, however, a crystalline dibasic acid, $\text{C}_{25}\text{H}_{34}\text{O}_{10}$ (III).



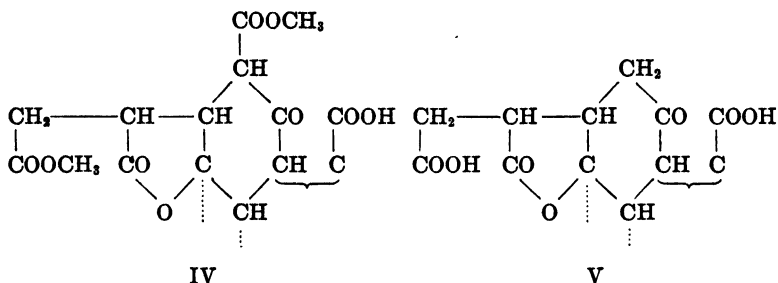
This dibasic acid on heating with acetic anhydride was easily converted into a crystalline anhydride. On attempting, however, thermic decomposition of the latter, no crystalline reaction product could be obtained. Apparently the stable carbomethoxyl group was involved in the decomposition, a fact which was indicated by analysis of the resulting resin for methoxyl. Fortunately, however, another method of approach became available.

It has already been shown³ that anhydroisostrophanthonic dimethyl ester on oxidation is degraded with the loss of its ketonic

² Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 820 (1927).

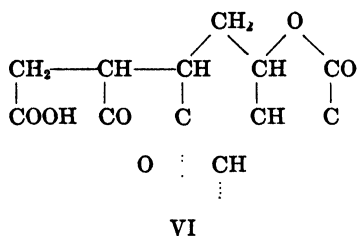
³ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **79**, 539 (1928).

carbon atom and the formation of a keto acid (IV), undephanthondiacid dimethyl ester.



This acid was shown to be a β -ketonic ester which readily undergoes the ketonic decomposition to form the keto acid, duodephanthondiacid (V).

In more recent work which will be described in the experimental part, the latter and even its dimethyl ester on reduction with catalytically activated hydrogen have given at once lactones (VI) due to the prompt lactonization of the free carboxyl group on the hydroxyl group produced by reduction of the carbonyl group. This ready lactonization practically restricts duodephanthondiacid



within the category of a γ - or δ -keto acid. Ring II of strophanthidin therefore must be either a 5- or a 6-membered ring. The decision between the two possibilities appeared to rest on the determination of whether duodephanthondiacid is a γ - or a δ -ketonic acid.

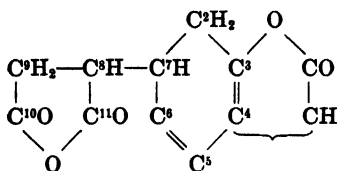
A possible method which was suggested for deciding this point was a study of the behavior of this acid on lactonization. γ -Ketonic acids on lactonization yield $\Delta^{\beta,\gamma}$ -unsaturated lactones while in the case of a δ -ketonic acid the product is a $\Delta^{\gamma,\delta}$ -un-

saturated lactone. It has been our experience that $\Delta^{\beta,\gamma}$ -unsaturated lactones which possess a free hydrogen in the α -position give the nitroprusside reaction.⁴ It was hoped, therefore, that the lactone obtained from duodephanthondiacid, when investigated from this standpoint, would yield the desired evidence. A positive Legal reaction would indicate a $\Delta^{\beta,\gamma}$ -lactone and therefore a γ -keto acid, while a negative reaction would indicate a $\Delta^{\gamma,\delta}$ -lactone or possibly the unlikely case of a $\Delta^{\beta,\gamma}$ -lactone with a quaternary α -carbon atom.

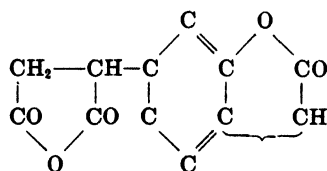
When an attempt was made to lactonize duodephanthondiacid directly by heating it above its melting point, only a non-crystalline reaction product was recovered. A crystalline substance, however, was readily obtained when the ketonic acid was heated with an acetic anhydride-acetyl chloride mixture. This substance proved to be of unexpected character and on analysis was found to have the formula $C_{21}H_{24}O_6$. The interpretation of the nature of this substance was aided by our previously reported study of the action of acetic anhydride-acetyl chloride on β -isostrophanthic lactone acid.⁵ It was found that the reagent had not only accomplished the lactonization desired but had also attacked another portion of the molecule. This consisted in the cleavage of the original lactone group with the formation of a substituted succinic anhydride with simultaneous loss of OH^1 to form an additional double bond. The resulting substance, therefore, was a doubly unsaturated lactone anhydride (VII). It was found to give no reaction with sodium nitroprusside. Although this result indicated that the substance is in all probability a $\Delta^{\gamma,\delta}$ -lactone and that duodephanthondiacid is therefore a δ -ketonic acid, caution was required because of the complicating presence of the extra double bond. The latter might conceivably have induced a secondary shift of the double bond produced by lactonization on the keto group. If the probable assumption is made that the two double bonds are conjugated then six arrangements of these bonds in Ring I are possible as shown in Formulæ VII to XII.

⁴ Jacobs, W. A., Hoffmann, A., and Gustus, E. L., *J. Biol. Chem.*, **70**, 1 (1926).

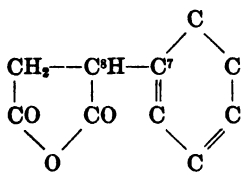
⁵ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **84**, 183 (1929).



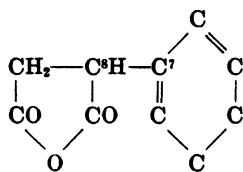
VII



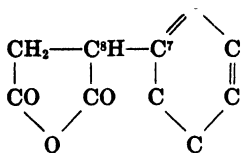
VIII



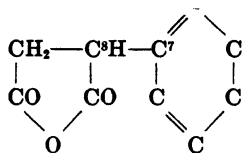
IX



X



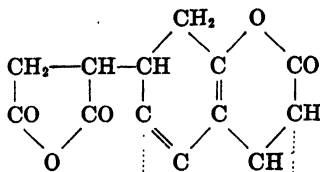
XI



XII

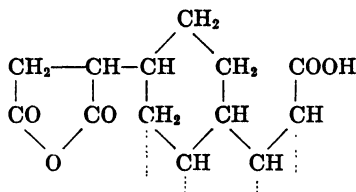
In Formulæ IX to XII a double bond is attached on one or the other side of carbon atom (7). In each of these cases there would therefore be a double bond β, γ to the proximal carbonyl of the anhydride group, and the intermediate carbon atom (8) would bear a hydrogen atom which should be active and give a positive Legal reaction. If such an assumption is acceptable, then these four arrangements are excluded from consideration because of the negative outcome of the Legal test with our substance. There remain, therefore, as possible arrangements free from this objection Formulæ VII and VIII, in which the other double bond is attached to carbon atom (3) which is the point of lactonization in question. If this were a γ -lactone then in both cases the α -carbon (if not quaternary) would bear an active hydrogen. Therefore, only if we are dealing with a $\Delta^{\gamma, \delta}$ -unsaturated lactone could we properly expect a negative reaction. If this argument is permissible then Formula XIII, which contains the most probable

arrangement for the double bonds, appears to be the most acceptable partial formula for this unsaturated lactone anhydride.



XIII

That a double bond is still at the point of lactonization is supported by the behavior of this substance on catalytic hydrogenation. The hydrogenation was found to go beyond the stage of the saturation merely of the two double bonds. It absorbed readily 3 mols of hydrogen and the resulting product proved to be a mixture apparently of isomeric desoxy acids. One of these anhydride acids (XIV) was obtained in crystalline form and on analysis gave figures in agreement with the required formulation, $C_{21}H_{30}O_5$.



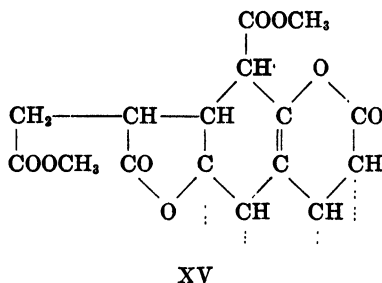
XIV

On saponification its anhydride group was readily opened with the formation of the tribasic acid, $C_{21}H_{32}O_6$, which was further characterized as the trimethyl ester.

If in the above lactonization of duodephanthondiacid the anhydride formation and consequent production of the additional double bond could have been prevented the evidence obtained and conclusions reached would have been based on fewer assumptions. This might have been accomplished by lactonization of the half-ester of this acid. The ester group would have prevented formation of the anhydride group with simultaneous production of the extra double bond. Unfortunately all attempts to realize this

plan by selective esterification of the one carboxyl group were unsuccessful.

When undephanthontriacid dimethyl ester was heated with acetic anhydride-acetyl chloride, it was readily converted into an *unsaturated lactone*, $C_{24}H_{30}O_8$ (XV), and because of the protecting ester group without the complication of anhydride formation and the production of a second double bond. The Legal test with this



substance was strongly positive. Undephanthontriacid dimethyl ester (IV) itself, however, also gives a Legal test as a β -ketonic ester. In the case of its unsaturated lactone the test may be given by the same active H atom which lies between the ester CO group and the double bond β, γ to the latter. Such a test is therefore inconclusive in this case.

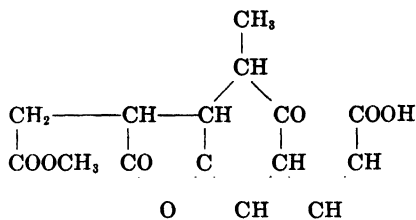
Fortunately, however, the recent correlation of periplogenin with strophanthidin⁶ has made it possible to obtain the desired confirmatory evidence by another method of approach. For this purpose we have prepared the substance analogous to undephanthontriacid dimethyl ester from periplogenin. In a recent communication⁷ a series of reactions has been described by which isoperiplogenic acid has been converted into anhydroisoperiplogonic methyl ester. This substance bears the same relationship to isoperiplogenin which anhydroisostrophanthonic dimethyl ester bears to isostrophanthidin. In the former ester a methyl group occupies the place of a carbomethoxyl group in the latter.

Anhydroisoperiplogonic methyl ester on oxidation with ozone yielded a degradation product which proved to be a ketonic acid,

⁶ Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **91**, 625 (1931).

⁷ Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **92**, 313 (1931).

$C_{23}H_{32}O_7$, with one less carbon atom, and has been designated *undeplogondiacid monomethyl ester* (XVI).



XVI

This ketonic acid, unlike the β -ketonic ester, undephantontriacid dimethyl ester, gives no Legal reaction since the ester group of the latter which is β to the CO group is replaced by a methyl group.

Undeplogondiacid monomethyl ester is readily lactonized by the acetic anhydride-acetyl chloride method. The resulting *unsaturated lactone* gives with nitroprusside a faint test which we regard as definitely atypical and therefore practically negative. It is possible that the faint atypical color is attributable to some impurity or to a shift in the double bond under the influence of alkali during the test.

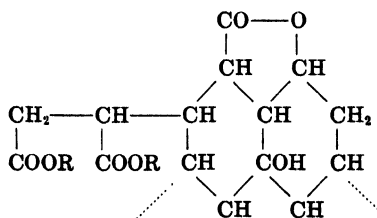
From these results all indications seem to point definitely to the conclusion that the ketonic acids which result from the degradation of anhydroisostrophanthonic dimethyl ester and anhydroisoperiplogonic methyl ester are δ -ketonic acids and that Ring II of both strophanthidin and periplogenin is therefore 6-membered.

This conclusion has recently received confirmation from another source. In previously reported work, Windaus, Westphal, and Stein⁸ have succeeded in oxidizing the secondary hydroxyl group of hexahydrodigitaligenin through the ketone to the dibasic acid, $C_{23}H_{34}O_6$, with ring cleavage. This acid on distillation yielded a "Brenzketon," $C_{22}H_{32}O_3$. From this result they have properly concluded that the ring of gitoxigenin and digitaligenin which bears the secondary hydroxyl group is 6- or possibly 7-membered. In the recent work from our laboratory on the correlation of both

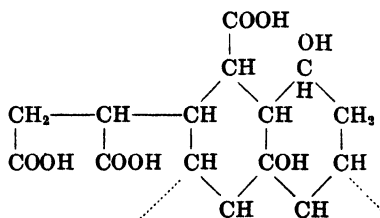
⁸ Windaus, A., Westphal, K., and Stein, G., *Ber. chem. Ges.*, **61**, 1847 (1928). Stein, G., Inaugural dissertation, University of Göttingen (1929).

digitoxigenin and gitoxigenin with strophanthidin and periplogenin,⁷ it has been established among other things that the secondary acylatable hydroxyl group is one of the features common to all of these aglucones. The results, therefore, of Windaus, Westphal, and Stein are at once applicable to the problem of the size of Ring II of strophanthidin. The only ring size which is compatible both with the formation of a pyroketone and with the ready lactonization of the above ketonic acids is a 6-membered ring. Ring II of the four cardiac aglucones must be, therefore, a substituted cyclohexane ring.

In the course of the work related to the problem of the size of Ring II several other methods of approach were attempted. Before the recent work leading to the preparation and study of undeplogondiacid monomethyl ester and its lactone, a plan had been formulated based on the use of β -isostrophanthic lactone acid.⁹ The opening of the labile lactone group of this substance with loss of hydroxyl^I has already been described,⁵ in which either acetic anhydride-acetyl chloride or methyl alcoholic hydrochloric acid was employed. Different stereoisomeric unsaturated dimethyl esters were obtained by the two methods. The ester obtained directly with methyl alcoholic hydrochloric acid, although unsaturated, resisted all efforts to hydrogenate its double bond catalytically. On the other hand the ester obtained through the intermediate anhydride was hydrogenated, although with difficulty, to the saturated desoxylactone dimethyl ester, $C_{25}H_{36}O_7$ (XVII). The stable lactone group of this substance involving carboxyl^I and hydroxyl^{III} was subsequently saponified with difficulty to the saturated *desoxytriacid*, $C_{23}H_{34}O_8$ (XVIII).



XVII

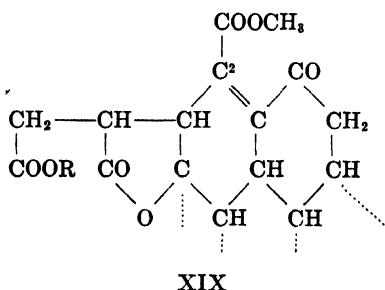


XVIII

⁹ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 829 (1927).

This acid was readily converted into the *trimethyl ester*. The secondary hydroxyl (OH^{II}) of this substance was easily oxidized to carbonyl with the formation of the *ketonic ester*. All further plans with this series of substances were frustrated at the next step when unsuccessful attempts were made to prepare the anhydro ketone by removal of hydroxyl^{II}. For this purpose the method was tried which has been repeatedly used with success for the preparation of the analogous anhydroisostrophanthonic dimethyl ester and anhydroisoperiplogonic methyl ester. This procedure as well as modifications have given only non-crystalline resins.

In the course of this work we have had occasion to prepare *β -isostrophanthonic dimethyl ester* from *β -isostrophanthic dimethyl ester*⁹ with chromic acid. As expected, this substance readily lost water to form an *anhydroisostrophanthonic dimethyl ester*. Examination of this substance proved it to be identical with the anhydro ester already prepared from *α -isostrophanthonic dimethyl ester*. The identity of the anhydro ketones from both the α and β series confirms the position of the double bond already suggested for this substance.³ Since the anhydro ketone gave no reaction with nitroprusside it had been suggested that the double bond formed by loss



of OH^{II} must shift to the position given in Formula XIX. Since the isomerism of α - and β -isostrophanthic acids and their derivatives has been shown to be due to the asymmetry of C^2 of the strophanthidin molecule, such asymmetry is lost by the double bond attached to it in anhydroisostrophanthonic dimethyl ester. The same anhydro ester should, therefore, be formed from the epimeric α - and β -isostrophanthonic esters.

EXPERIMENTAL

Part A

Undephanthotriacid Dimethyl Ester—A great improvement in the yield of this substance over that previously reported³ in the oxidation of anhydroisostrophanthonic dimethyl ester was obtained by the use of ozone as the oxidizing agent. A stream of ozonized oxygen (about 1 to 1.5 per cent) was passed through a solution of 3 gm. of the dimethyl ester in 70 cc. of acetic acid kept at 20° at the rate of 1 liter per minute for 1 hour. The clear solution was concentrated under diminished pressure to small volume. After the addition of 300 cc. of water, the mixture was heated for a time on the water bath to decompose any ozonides. During this operation all with the exception of a small amount of oil dissolved. On being cooled, undephanthotriacid dimethyl ester gradually crystallized. The yield of crude material varied from 2 to 2.5 gm. Additional substance was recovered by extraction of the aqueous filtrate, and this was suitable for conversion into duodephanthondiacid. After recrystallization from methyl alcohol it formed leaflets which melted at 167–168°, somewhat lower than was observed with the substance previously reported (179–180°).

3.160 mg. substance: 1.963 mg. H₂O, 7.168 mg. CO₂.

C₂₄H₃₂O₉. Calculated. C 62.03, H 6.95

Found. " 61.86, " 6.95

In view of the discrepancy in melting points, the identity of the substances obtained by both methods was checked by a comparison of the same derivatives prepared from each. The trimethyl ester obtained by the action of diazomethane on the ozone product melted at 149–151° and was again somewhat lower than that previously reported for the trimethyl ester, *viz.* 154.5–155.5°. This melting point, however, was found to be easily depressed by slight impurities. A mixture of esters from both sources melted at 153–155°. The rotations of both samples were practically negligible.

The results of the analysis on the more recent trimethyl ester were as follows:

3.205 mg. substance: 2.095 mg. H₂O, 7.370 mg. CO₂.

C₂₅H₃₄O₉. Calculated. C 62.73, H 7.17

Found. " 62.71, " 7.30

In all other respects their properties were identical. A phenylhydrazone prepared from the new trimethyl ester melted at 197° as previously reported and showed no depression when mixed with the original phenylhydrazone.

4.761 mg. substance: 2.950 mg. H_2O , 11.425 mg. CO_2 .

$C_{31}H_{40}O_8N_2$. Calculated. C 65.46, H 7.10
Found. " 65.39, " 6.93

Duodephanthondiacid—Here again the preparation of this acid from the above dimethyl ester was greatly improved by the substitution of piperidine for 0.1 N NaOH as previously described.³ 2.5 gm. of crude undephanthontriacid dimethyl ester, as directly obtained, were dissolved in a mixture of 12.5 cc. of piperidine and 25 cc. of water. The solution was refluxed preferably in an atmosphere of nitrogen for 16 hours after which time the solution no longer gave a nitroprusside reaction. After concentration to dryness under diminished pressure the residue was redissolved in water. On acidification to Congo red the acid gradually crystallized. The amount obtained was 60 to 80 per cent of the weight of the dimethyl ester employed. After recrystallization from acetone it melted with effervescence at 264° . In all respects the substance proved to be identical with that previously described.

4.260 mg. substance: 2.835 mg. H_2O , 10.041 mg. CO_2 .

$C_{21}H_{28}O_7$. Calculated. C 64.25, H 7.20
Found. " 64.28, " 7.45

For further characterization the dimethyl ester was prepared, as previously described, with diazomethane. The ester is characterized by its great crystallizing powers. It separated readily from methyl alcohol as sparingly soluble prisms which melted at 166° , and showed no depression when mixed with the ester originally described.

4.332 mg. substance: 2.988 mg. H_2O , 10.445 mg. CO_2 .

$C_{22}H_{32}O_7$. Calculated. C 65.67, H 7.68
Found. " 65.76, " 7.72

The ester was also prepared by gently heating for 3 minutes at 70° a solution of the acid in 0.5 per cent dry methyl alcoholic hydrochloric acid. Longer heating resulted in a more deep-seated reaction which gave rise to obscure non-crystalline products.

Duodephanthondiacid Dimethyl Ester Ketazine—A solution of 0.1 gm. of duodephanthondiacid dimethyl ester in a few cc. of methyl alcohol was treated with an excess (over 3 mols) of a 3 per cent hydrazine solution which had been previously neutralized with acetic acid. On standing at ordinary temperature the mixture gradually deposited in excellent yield fine needles of the ketazine. After recrystallization from methyl alcohol it melted at 184°.

4.260 mg. substance: 2.935 mg. H₂O, 10.305 mg. CO₂.
 6.182 " " : 0.194 cc. N (761 mm., 24°).
 C₄₆H₆₄O₁₂N₂. Calculated. C 65.98, H 7.71, N 3.35
 Found. " 65.98, " 7.70
 " " 3.61

Reduction of Duodephanthondiacid—0.35 gm. of carefully recrystallized duodephanthondiacid in alcoholic solution was shaken with hydrogen and 0.1 gm. of Adams and Shriner's platinum oxide catalyst. After the initial reduction of the catalyst 1 mol of hydrogen was absorbed within 20 minutes, and then the reaction stopped. After concentration the reduction product readily crystallized. This substance proved to be the dilactone acid due to simultaneous lactonization. It formed prisms and narrow platelets from alcohol which melted at 253°.

4.380 mg. substance: 2.935 mg. H₂O, 10.735 mg. CO₂.
 C₂₁H₂₈O₆. Calculated. C 66.99, H 7.50
 Found. " 66.84, " 7.50

In another experiment the reaction was found to take a different course when a sample of duodephanthondiacid of different origin was employed. This material had been prepared by the previously described method in which undephanthontriacid dimethyl ester obtained by the permanganate oxidation of anhydroisostrophanthonic dimethyl ester was decomposed by refluxing with 0.1 N alkali. 0.7 gm. of this material in alcoholic solution was reduced with 50 mg. of platinum oxide catalyst and hydrogen. After concentration of the filtrate from the catalyst a crystalline residue was obtained which after recrystallization from a small volume of methyl alcohol yielded flat needles or narrow leaflets which melted at 221°. On recrystallization from methyl alcohol this

melting point was raised to 234°. Analysis and titration showed that the substance was the *hydroxy acid*.

4.642 mg. substance: 3.120 mg. H_2O , 10.895 mg. CO_2 .

$\text{C}_{21}\text{H}_{30}\text{O}_7$. Calculated. C 63.93, H 7.67

Found. " 64.01, " 7.52

16.467 mg. of substance were dissolved in 1 cc. of alcohol and directly titrated with 0.1 N NaOH against phenolphthalein. Found, 0.795 cc. Calculated for 2 equivalents, 0.835 cc. The above solution was treated with 3 cc. of 0.1 N NaOH, refluxed for 4 hours, and then titrated back. Found, 0.460 cc. or a total of 1.255 cc. Calculated for 3 equivalents, 1.252 cc.

We have not investigated further the reason for the formation of the intermediate hydroxy acid in this case. Later attempts to duplicate the preparation of the hydroxy acid (or the hydroxy ester from the dimethyl ester as given below) always gave the dilactone. This fact was of much greater consequence in our problem.

Reduction of Duodephanthondiacid Dimethyl Ester—In repeated experiments with the dimethyl ester no duodephanthondiacid dimethyl ester could be obtained since lactonization with cleavage of methyl alcohol proceeded too quickly. The substance which resulted was the dilactone monomethyl ester.

0.27 gm. of the dimethyl ester was suspended in methyl alcohol and shaken with 0.1 gm. of platinum oxide and hydrogen. The sparingly soluble ester dissolved within about 20 minutes after the absorption of 1 mol of H_2 . Concentration of the filtrate from the catalyst yielded the sparingly soluble monomethyl ester. Recrystallized from dilute acetone, it formed needles which melted at 240°. It is sparingly soluble in methyl and ethyl alcohols at ordinary temperature but is readily soluble in chloroform and acetone.

5.090 mg. substance: 3.585 mg. H_2O , 12.596 mg. CO_2 .

5.300 " " : 3.260 " AgI.

$\text{C}_{23}\text{H}_{30}\text{O}_8$. Calculated. C 67.65, H 7.75, OCH_3 7.95

Found. " 67.48, " 7.88

" " 8.12

15.819 mg. of substance were refluxed in 2 cc. of alcohol and 3 cc. of 0.1 N NaOH for 3 hours and then titrated back against phenol-

phthalein. Calculated for 3 equivalents, 1.216 cc. Found, 1.178 cc.

Unsaturated Lactone Anhydride, $C_{21}H_{24}O_5$, from Duodephanthondiacid—The attempt by means of methyl alcoholic hydrochloric acid to open the lactone group of duodephanthondiacid with simultaneous dehydration and esterification in order to form an unsaturated substituted succinic ester was unsuccessful, due to the non-crystalline character of the reaction products. Success was obtained by the use of acetic anhydride and acetyl chloride which, while giving an unsaturated substituted succinic anhydride, caused simultaneous lactonization of the carboxyl group on the carbonyl group.

2.7 gm. of duodephanthondiacid were heated in a sealed tube at 80° with a mixture of 30 cc. of acetic anhydride and 3 cc. of acetyl chloride. The sparingly soluble substance gradually dissolved when the mixture was shaken. After solution the heating was continued for 18 hours. On concentration under diminished pressure a crystalline residue remained. This was redissolved in chloroform and the solution was extracted with dilute sodium carbonate solution, then with water, and was finally dried. On careful addition of petroleic ether to the concentrated solution, platelets of the unsaturated lactone anhydride separated. The yield was 2.05 gm. When recrystallized from dilute acetone the substance forms long narrow plates which melted at 242° after preliminary sintering. It is readily soluble in chloroform and acetone and very sparingly soluble in methyl and ethyl alcohols, ether, and benzene. The substance does not give the Legal reaction with nitroprusside.

5.167 mg. substance: 3.250 mg. H_2O , 13.393 mg. CO_2 .

$C_{21}H_{24}O_5$. Calculated. C 70.76, H 6.79

Found. " 70.68, " 7.03

The anhydride nature of the substance was shown by its behavior toward alkali. Although insoluble in dilute sodium carbonate solution it rapidly dissolved in dilute NaOH solution. On being boiled with alkali, 3 equivalents were consumed, 2 by the anhydride group and 1 by the lactone group.

15.102 mg. of substance were refluxed with 2 cc. of alcohol and 3 cc. of 0.1 N NaOH for 3 hours and then titrated back against

phenolphthalein. Calculated for 3 equivalents, 1.272 cc. Found, 1.258 cc.

Hydrogenation of the Unsaturated Lactone Anhydride, $C_{21}H_{24}O_5$, to the Saturated Desoxy Acid Anhydride, $C_{21}H_{30}O_5$ —0.52 gm. of the unsaturated lactone anhydride was hydrogenated in acetic acid solution with 50 mg. of platinum oxide catalyst. A rapid absorption occurred which practically stopped after 20 minutes. After deduction for the reduction of the catalyst, the absorption amounted to 105 cc. (0° , 760 mm.). Calculated for 3 mols, 99 cc. On concentration to dryness under diminished pressure a glassy residue remained which crystallized rapidly when treated with dry ether. After standing, the crystals were collected with ether. On careful addition of petroleic ether to the solution in chloroform, the substance separated as needles which melted at 173° after slight preliminary softening. The yield of the crystalline acid was approximately one-third of the weight of the starting material. The major portion of reaction product remained in solution and on concentration remained as a resin which could not be made to crystallize. This material was unquestionably a mixture of low melting isomers which were simultaneously formed during the hydrogenation.

The acid character of the reaction product was readily seen by its ready solution in dilute carbonate in contradistinction to the unsaturated lactone anhydride. It is soluble in the usual organic solvents with the exception of petroleic ether. For analysis the substance was dried at 100° and 15 mm.

4.828 mg. substance: 3.620 mg. H_2O , 12.360 mg. CO_2 .

$C_{21}H_{30}O_5$. Calculated. C 69.57, H 8.35

Found. " 69.82, " 8.39

Saturated Triacid, $C_{21}H_{32}O_6$ —On warming the above anhydride acid with dilute alkali the anhydride group was readily saponified. On acidification an amorphous mass separated which gradually crystallized as delicate needles. The tribasic acid was recrystallized by the careful dilution of its solution in a small volume of acetone. It formed minute delicate needles which were difficult to filter. It did not exhibit a sharp melting point but slowly softened to a resin as the temperature rose above 120° .

For analysis the substance was dried at 100° and 15 mm.

5.197 mg. substance: 4.110 mg. H₂O 12.680 mg. CO₂.
 $C_{21}H_{32}O_6$. Calculated. C 66.27, H 8.48
 Found. " 66.55, " 8.85

Saturated Triacid Trimethyl Ester—The above triacid in ether solution was treated with diazomethane. On concentration a colorless oil remained which finally crystallized after long standing. Because of its solubility in most organic solvents petroleic ether was found most suitable for its collection and recrystallization. From its concentrated solution in this solvent it slowly separated as needles which melted at 62°.

4.920 mg. substance: 3.880 mg. H₂O, 12.300 mg. CO₂.
 3.830 " " : 6.268 " AgI.
 $C_{24}H_{38}O_6$. Calculated. C 68.20, H 9.07, OCH₃ 22.01
 Found. " 68.18, " 8.82, " 21.62

Unsaturated Lactone from Undephanthontriacid Dimethyl Ester—1.5 gm. of undephanthontriacid dimethyl ester were heated in a sealed tube with 30 cc. of acetic anhydride and 3 cc. of acetyl chloride at 80° for 15 hours. After concentration under diminished pressure a crystalline residue remained. This was dissolved in chloroform and the solution was extracted with dilute carbonate and then water. The unsaturated lactone was obtained on concentration of the chloroform solution. On recrystallization from acetone it formed needles which melted at 199°. Although it may be recrystallized from methyl alcohol this was found to be complicated by the ready cleavage of the unsaturated lactone group to form the neutral trimethyl ester of undephanthontriacid.

For analysis the substance was dried at 100° and 15 mm.

3.765 mg. substance: 2.355 mg. H₂O, 8.925 mg. CO₂.
 3.903 " " : 4.100 " AgI.
 $C_{24}H_{30}O_8$. Calculated. C 64.54, H 6.78, OCH₃ 13.90
 Found. " 64.65, " 7.00
 " " 13.87

The unsaturated lactone gives a Legal reaction which is more pronounced than in the case of undephanthontriacid dimethyl ester.

Undepgondiacid Monomethyl Ester—0.25 gm. of anhydroiso-

periplogonic methyl ester⁷ dissolved in 25 cc. of acetic acid was oxidized by ozone under the conditions used with anhydroisostrophanthonic dimethyl ester. After removal of the solvent under diminished pressure, the resinous residue was treated with water and heated on the bath to decompose any ozonide. On cooling and standing, the oxidation product gradually crystallized. On careful dilution of the acetone solution it gradually formed a mass of delicate needles which melted at 182–184°. The substance was completely soluble in dilute ammonia. Contrary to the analogous strophanthidin derivative it gave no reaction with nitroprusside.

3.627 mg. substance: 2.457 mg. H₂O, 8.680 mg. CO₂.

3.755 " " : 2.137 " AgI.

C₂₃H₃₂O₇. Calculated. C 65.68, H 7.68, OCH₃ 7.38

Found. " 65.27, " 7.58

"

" 7.52

Unsaturated Lactone, C₂₃H₃₀O₆, from Undeplogondiacid Monomethyl Ester—The previous keto acid was heated at 80° for 16 hours in 10 parts of a mixture of acetic anhydride and acetyl chloride (10:1). After removal of the reagent under reduced pressure the residue was dissolved in chloroform. The solution was washed with dilute carbonate. The concentrated chloroform solution crystallized readily when treated with ether. On recrystallization from chloroform-ether it separated as broad prismatic needles which melted at 235–236°.

4.155 mg. substance · 2.750 mg. H₂O, 10.425 mg. CO₂.

C₂₃H₃₀O₆. Calculated. C 68.61, H 7.52

Found. " 68.43, " 7.41

This unsaturated lactone gives a faint, slowly developing, atypical Legal reaction which is not comparable with that usually given by $\Delta^{\beta,\gamma}$ -lactones or by the analogous unsaturated lactone from undephanthontriacid dimethyl ester.

Part B

Saponification of the Saturated Lactone Dimethyl Ester, C₂₅H₃₆O₇, to the Saturated Triacid, C₂₃H₃₄O₈—The saturated lactone dimethyl ester, C₂₅H₃₆O₇, is a substance previously described.⁵ Its origin

was as follows: β -Isostrophanthic lactone acid⁹ yielded an unsaturated anhydroanhydride acetate, $C_{25}H_{30}O_7$, with acetic anhydride-acetyl chloride. This on saponification gave the dibasic anhydrolactone acid, $C_{23}H_{30}O_7$, which was converted into the so called β -dimethyl ester, $C_{25}H_{34}O_7$. This ester on hydrogenation gave the saturated lactone dimethyl ester which was used in the following experiments.

The complete saponification of the lactone ester to the triacid was complicated by the resistance to hydrolysis of the stable lactone group which is characteristic of substances of the β -isostrophanthic lactone acid series and the sparing solubility of the sodium salt of the dibasic acid first formed on saponification of the two methyl ester groups. Depending upon the conditions employed either result could be obtained.

0.1 gm. of the lactone dimethyl ester was refluxed in a mixture of 6 cc. of 10 per cent NaOH and 6 cc. of absolute alcohol. Solution was complete in 15 minutes. If the heating was continued longer under these conditions with the hope of saponifying the lactone group this was prevented by the gradual separation of the sparingly soluble sodium salt of the dibasic lactone acid. On dilution the salt redissolved and on subsequent acidification the free acid separated as platelets. From dilute acetone it formed diamond-shaped plates which frothed up at 258° after preliminary softening.

For analysis the substance was dried at 100° and 15 mm.

4.305 mg. substance: 0.322 mg. H_2O .

$C_{23}H_{32}O_7 \cdot 2H_2O$. Calculated. H_2O 7.89. Found. 7.48

3.983 mg. anhydrous substance: 2.770 mg. H_2O , 9.630 mg. CO_2 .

$C_{23}H_{32}O_7$. Calculated. C 65.67, H 7.68

Found. " 65.93, " 7.78

For complete saponification to the tribasic acid the following procedure was used.

0.2 gm. of the lactone dimethyl ester was refluxed in a mixture of 5 cc. of pyridine, 15 cc. of 2 N NaOH, and 10 cc. of water for 4 hours. On acidification the acid separated first as a jelly which gradually crystallized. On recrystallization from dilute acetone it separated as compact masses which contained solvent and

effervesced at 225°. On drying at 100° and 15 mm. water was not completely removed.

4.704 mg. substance: 3.315 mg. H₂O, 10.700 mg. CO₂.

C₂₈H₃₄O₈ · $\frac{1}{2}$ H₂O. Calculated. C 61.70, H 7.90

Found. " 62.04, " 7.88

Trimethyl Ester, C₂₆H₄₀O₈—The ester, prepared with diazomethane, formed delicate needles from dilute methyl alcohol which melted at 199° and were easily soluble in the alcohols and acetone and appreciably in ether.

For analysis it was dried at 100° and 15 mm.

4.918 mg. substance: 3.710 mg. H₂O, 11.710 mg. CO₂.

4.360 " " : 6.250 " AgI.

C₂₆H₄₀O₈. Calculated. C 64.97, H 8.40, OCH₃ 19.37

Found. " 64.95, " 8.44, " 18.92

Keto Ester, C₂₆H₃₈O₈—The above trimethyl ester was oxidized at ordinary temperature in acetic acid solution with Kiliani chromic acid solution. When recrystallized from methyl alcohol the ketone formed needles which melted at 220°.

4.470 mg. substance: 3.160 mg. H₂O, 10.740 mg. CO₂.

C₂₆H₃₈O₈. Calculated. C 65.24, H 8.01

Found. " 65.52, " 7.91

Repeated attempts to convert this substance into an anhydro compound resulted only in the formation of non-crystalline resins.

The oxime formed needles from methyl alcohol which melted at 210°.

4.390 mg. substance: 3.190 mg. H₂O, 10.208 mg. CO₂.

C₂₆H₃₉O₈N. Calculated. C 63.25, H 7.97

Found. " 63.37, " 8.13

β-Isostrophanthonic Dimethyl Ester—This was prepared from β-isostrophanthic dimethyl ester exactly as described in our earlier work with the α-ester.¹⁰ The keto ester formed long narrow plates from methyl alcohol which melted at 248–250°. It is easily soluble

¹⁰ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 819 (1927).

in chloroform and acetone, sparingly soluble in the alcohols, and insoluble in ether.

4.115 mg. substance: 2.730 mg. H_2O , 9.785 mg. CO_2 .

$\text{C}_{25}\text{H}_{34}\text{O}_8$. Calculated. C 64.90, H 7.41

Found. " 64.85, " 7.42

The oxime crystallized from dilute methyl alcohol as needles which sintered above 128° and frothed up at 190° , then resolidified and remelted at $215\text{--}217^\circ$.

4.198 mg. substance: 2.890 mg. H_2O , 9.622 mg. CO_2 .

$\text{C}_{25}\text{H}_{35}\text{O}_8\text{N}$. Calculated. C 62.86, H 7.39

Found. " 62.51, " 7.71

β -Isostrophanthonic ester was dehydrated as previously described in the case of the α -isomer,¹⁰ with the exception that it is preferable to substitute methyl alcohol for ethyl alcohol to avoid "Umesterung." The anhydro compound formed rhombs from methyl alcohol which melted at $205\text{--}206^\circ$ and were indistinguishable from the previously described anhydro- α -isostrophanthonic dimethyl ester. The melting point of the latter which was previously reported at 210° was found to vary. It was found after recrystallization to be 206° . No depression was obtained with the mixture.

The substance from the β -series showed a rotation

$$[\alpha]_D^{20} = +72^\circ \text{ (c = 0.555 in pyridine).}$$

The rotation previously reported with the substance obtained from the α -series was:

$$[\alpha]_D^{25} = +74^\circ \text{ (c = 1.023 in pyridine).}$$

4.205 mg. substance: 2.720 mg. H_2O , 10.403 mg. CO_2 .

$\text{C}_{25}\text{H}_{32}\text{O}_7$. Calculated. C 67.53, H 7.26

Found. " 67.47, " 7.24

Part C

The Oxidative Cleavage of Desoxy- α -Isostrophanthonic Dimethyl Ester to the Dibasic Acid, $\text{C}_{25}\text{H}_{34}\text{O}_{10}$ —In more recent experiments on the preparation of desoxyisostrophanthonic dimethyl ester² by hydrogenation of anhydro- α -isostrophanthonic dimethyl ester, the more sparingly soluble isomer which separated as stout prisms or rhombs was always found to be the preponderating reaction

product. A number of modifications of the method of hydrogenation did not alter the result. This isomer, for which a rotation was found of $[\alpha]_D = -4^\circ$ ($c = 0.7$ in pyridine), was found on rechecking to give $[\alpha]_D = +8^\circ$ ($c = 0.5$ in pyridine). On oxidation of this ketonic ester with CrO_3 no crystalline reaction product could be obtained. The other more soluble isomer which was always formed in much smaller amount gave a crystalline dibasic acid as follows:

0.5 gm. of the desoxy keto ester of $[\alpha]_D = -57^\circ$ ($c = 0.7$ in pyridine) was dissolved in a mixture of 10 cc. of acetic acid and 1 cc. of H_2O . 2 cc. of Kiliani CrO_3 solution were added and the mixture was heated at 70° for 1 hour. The diluted mixture was extracted with chloroform. The extract after repeated washings with water to remove excess acetic acid was extracted with dilute ammonia. The concentrated aqueous solution on acidification gave a copious separation of the acid which gradually crystallized. This dibasic acid formed needles from dilute acetone which melted at $191\text{--}193^\circ$.

4.692 mg. substance: 2.985 mg. H_2O , 10.512 mg. CO_2 .

4.235 " " : 4.000 " AgI.

$\text{C}_{25}\text{H}_{14}\text{O}_{10}$.	Calculated	C 60.70, H 6.93, OCH_3 12.55
	Found.	" 61.10, " 7.12
	"	" 12.48

When an attempt to distil this acid under very low pressure was made in order to obtain a possible pyroketone, decomposition occurred and no crystalline reaction product could be obtained.

A small amount of unchanged keto ester was recovered from the neutral fraction of the above oxidation mixture.

Anhydride, $\text{C}_{25}\text{H}_{32}\text{O}_9$ —The above dibasic acid was heated at 80° for 16 hours in a sealed tube with 10 parts of an acetic anhydride-acetyl chloride mixture (10:1). On evaporation under diminished pressure a residue remained which readily crystallized under ether. The anhydride separated on addition of ether to its chloroform solution as micro leaflets which melted at 256° with decomposition.

3.488 mg. substance: 2.192 mg. H_2O , 8.037 mg. CO_2 .

3.773 " " : 3.800 " AgI.

$\text{C}_{25}\text{H}_{32}\text{O}_9$.	Calculated.	C 62.99, H 6.77, OCH_3 13.03
	Found	" 62.84, " 7.03
	"	" 13.30

THE USE OF THE MOLISCH (α -NAPHTHOL) REACTIONS IN THE STUDY OF SUGARS IN BIOLOGICAL FLUIDS

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The α -naphthol (Molisch) reaction for sugars has been neglected as a possible basis for a quantitative method. As a ring test, the only form in which it is ever described, it is very crude. The violet colors produced at the surface of contact of concentrated sulfuric acid and a sugar solution containing α -naphthol are often obscured by charring. The lack of control of the concentration of sugar tends to give results which appear qualitatively the same for all carbohydrates.

The present paper describes a modification of the test and the results which it gives with various sugars and biological fluids as observed with a spectrophotometer. It will deal only with the qualitative nature of the reaction. The application of the Molisch reaction to a method for estimating very small quantities of carbohydrates will be described in future communications.

The chemical basis of the α -naphthol test is the production of furfural derivatives on heating carbohydrates with strong acids and the condensation of these furfuraldehydes with α -naphthol to give colored compounds. In the Molisch ring-test concentrated sulfuric acid is used both to produce the furfural derivatives and as the condensing agent.

For ordinary laboratory purposes the following procedure will give a delicate and satisfactory test. The color resulting will be evenly distributed throughout the test mixture and there will be no interference by charring.

The Qualitative Molisch Test

0.5 cc. of the solution to be tested is mixed with 5 cc. of cold 75 per cent (by volume) sulfuric acid. 3 drops of a 3 per cent alco-

holic solution of α -naphthol are added and thoroughly mixed with the acid mixture. A yellow color is produced by the addition of the naphthol. The mixture is warmed, preferably in a water bath, to about 80° . According to the chemical structure and concentration of the sugar present, a red to blue-violet color will be produced throughout the whole mixture.

The colored solutions fade on long standing and acquire a yellow-green fluorescence. Dilution with water destroys the color when the acid concentration is reduced below about 55 per cent by volume. If the concentration of sugar be relatively high, the color will be violet to blue-violet no matter what sugar is used, and on standing the pigment will collect at the surface of a yellowish solution. No organic solvent has yet been found which will extract the color produced in this test. Ethyl and amyl alcohols, benzene, xylene, acetone, and ether all destroy the color.

For the purpose of a spectrophotometric study of the Molisch test more rigid conditions were maintained. 1 cc. of sugar solution was mixed, in a 60 cc. flask, with 10 cc. of cold 75 per cent sulfuric acid. 0.1 cc. of a 3 per cent solution of α -naphthol in 95 per cent alcohol was added and the flask shaken to give thorough mixing. The mixture was then heated for exactly 20 minutes in a water bath at 45° . After being cooled (by placing the flask in cold water) the colored solution was transferred to an observation tube 3 cm. long. The absorption of light in the visible region between 4800 \AA. and 6400 \AA. was measured by a Bausch and Lomb spectrophotometer, consisting of a constant deviation type spectrometer and a Martens photometer. The light source was a 250 watt Mazda lamp. The results were expressed as the extinction

coefficient, $\log \frac{\text{incident light}}{\text{transmitted light}}$. In this preliminary qualitative study the concentrations of the carbohydrate solutions were not controlled further than was necessary to produce test mixtures of a color intensity measurable by the apparatus used.

Examination of the Molisch reactions of the simpler sugars showed that the final colored mixtures always have at least one absorption band in the visible region of the spectrum (4800 \AA. to 6400 \AA.). The position of maximum light absorption in these bands depends primarily upon the chemical nature of the sugar examined. The aldoses tested all showed a maximum of absorp-

tion between 4800 Å. and 5100 Å., whereas the ketoses (all of which, however, contained levulose in some form in their molecules) showed the maximum between 5700 Å. and 5800 Å. with the exception of melezitose. Furfuraldehyde itself gave an absorption

TABLE I

Position of Maximum Absorption for the Molisch Tests of Simple Sugars

Group 1		Group 2	
Sugar	Å.	Sugar	Å.
<i>d</i> -Arabinose.....	4900	<i>d</i> -Fructose.....	5700-5800
<i>d</i> -Xylose.....	4900	Sucrose.....	5700-5800
<i>d</i> -Glucose.....	4800-4900	Invert sugar.....	5700-5800
<i>d</i> -Mannose.....	5000-5100	Inulin.....	5700-5800
<i>d</i> -Galactose.....	4900	Melezitose.....	5500-5600
Maltose.....	4800		
Lactose.....	4900		
Maximum absorption.	4800-5100		5700-5800

TABLE II

Molisch Test

Sugar treated with Ca(OH) ₂	Absorption maximum before treatment	Absorption maximum after treatment
	Å.	Å.
<i>d</i> -Xylose.....	4900	5500-5600
<i>d</i> -Arabinose.....	4900	5600-5700
<i>d</i> -Galactose.....	4900	5500-5600
Lactose.....	4900	5600-5700

maximum at 5400 Å. to 5500 Å. Table I gives the positions of the maximum for the Molisch tests of the simple sugars examined.

Further evidence of the dependence of the position of the absorption maximum in the Molisch color reaction upon the constitution of the sugar was obtained by use of the Lobry de Bruyn transformation of aldoses and ketoses in the presence of alkali. Recently Montgomery and Hudson (1) prepared lactulose, a galactose-fructose complex, by treating lactose with saturated Ca(OH)₂ solution. With their method, solutions of arabinose, xylose,

galactose, and lactose have been subjected to the action of $\text{Ca}(\text{OH})_2$ for 24 hours. After treatment with 10 per cent H_2SO_4 and removal of CaSO_4 by filtration, the filtrates were examined by the usual Molisch test. In all cases one band was prominent and its absorption maximum was between 5500 Å. and 5800 Å. The solutions tested undoubtedly contained aldoses, but, as will be shown in a further communication on a quantitative Molisch test, a given quantity of ketose gives a far more intense Molisch test than does its equivalent of aldose. Hence we have the absence of maxima for aldoses in the Group 2 tests of Table I with sucrose, invert sugar, and melezitose and in the third column of Table II.

A series of simple derivatives of sugars was examined qualitatively. The derivatives and methods of preparation were as follows:

Phenylglucosazone—Glucose solution was heated with phenylhydrazine hydrochloride and sodium acetate in the boiling water bath. The precipitated osazone was filtered off and recrystallized twice from 95 per cent ethyl alcohol. It was used in the Molisch test in alcoholic solution.

Glucosone—Phenylglucosazone was heated in a water bath for about 10 minutes with concentrated HCl and the resulting solution used in the Molisch test.

Fructosamine—An alcoholic solution of glucosazone was boiled with zinc dust and glacial acetic acid. The undissolved zinc was filtered off, the filtrate cooled and used in the Molisch test.

Mannosamine (?)—Mannose phenylhydrazone was prepared in the usual manner. The product was washed thoroughly with distilled water and 95 per cent ethyl alcohol. Microscopic examination showed it to be free from glucosazone. An aqueous suspension of the hydrazone was boiled with zinc dust and acetic acid, filtered, and the filtrate used.

Phenylhydrazine hydrochloride and aniline sulfate, in concentrations far greater than could be produced by the action of acid on glucosazone, gave no color in the Molisch test.

The above sugar derivatives gave Molisch test solutions having the following absorption maxima.

Absorption Maxima

Phenylglucosazone.....	4800-4900 Å. and 5600-5700 Å.
Glucosone.....	4800-4900 " " 5600-5700 "
Fructosamine.....	5600-5700 "
Mannosamine (?).....	5000-5100 "

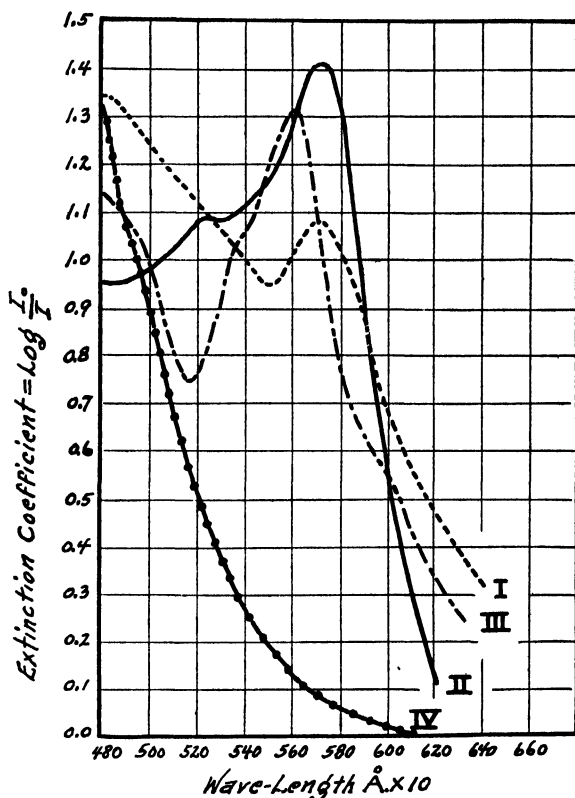


FIG. 1. The spectrum of Molisch tests given by normal blood (Curve I), normal spinal fluid (Curve II), normal urine (Curve III), and saliva (Curve IV).

It is probable that the phenylglucosazone was converted to the osone by the 75 per cent sulfuric acid used in the Molisch test. The osone, being both aldehyde and ketose, gave absorption maxima for both types of Molisch test. The "osamines" gave the maxima typical of the sugars from which they were formed.

From the above results it appears certain that in the Molisch reaction, aldoses give red colorations with an absorption maximum between 4800 Å. and 5100 Å. while ketoses give red-violet and

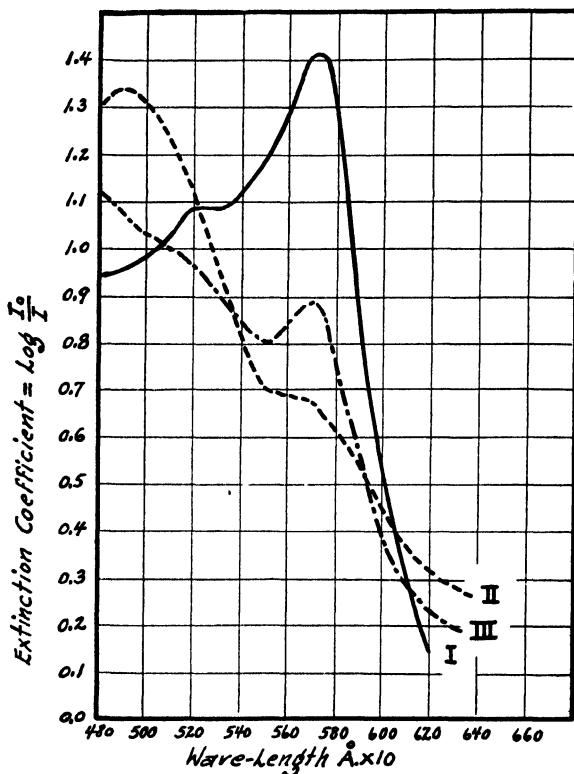


FIG. 2. The spectrum of the Molisch test given by spinal fluid: (Curve I) normal spinal fluid; (Curve II) fluid from a fatal case of meningococcic meningitis, taken 1 hour before death; (Curve III) fluid from a case of meningococcic meningitis during recovery. Note the change in absorption between 5600 Å. and 5800 Å.

blue-violet colors having an absorption maximum between 5500 Å. and 5800 Å.

It seems possible that a quantitative study of the rarer simple sugars would show even closer connection between the position of the maximum of light absorption of the Molisch test and the chemical configuration in the sugars.

With the position of maximum light absorption in the Molisch test being used as a criterion of the presence of aldose or ketose, certain normal and pathological body fluids have been examined. They were as follows:

Normal Blood Filtrates—Filtrates from human or rabbit blood were prepared by both the Folin-Wu tungstate method and the trichloroacetic acid method.

TABLE III

Comparison of Results with Molisch Test and That of Tashiro and Tietz

	Levulose equivalent to ketose present	
	Molisch test	Tashiro and Tietz test
	<i>mg. per cent</i>	<i>mg. per cent</i>
Normal human blood.....	9-14	Negative
“ rabbit “	10-17	
Diabetic human blood.....	13-21	
Normal urine.....	40	About 60
Normal human spinal fluid.....	3.0-3.5	
Spinal fluid from fatal case of meningococcic meningitis.....	1.0	
Spinal fluid during recovery from meningococcic meningitis.....	1.4	
Adenosine (British Drug Houses, Ltd.)....	Typical ketose spectrum	Ketose test
Thymonucleic acid.....		
Starch, 1 per cent aqueous paste made by boiling.....	About 11	
		Test given by 8 per cent paste

Normal spinal fluid, obtained by lumbar puncture and centrifuged before use.

Spinal fluids from a case of meningococcic meningitis (1 hour before death) and from another case during recovery. These also were centrifuged.

Normal human saliva, tested after filtration.

Normal Urine—To remove substances which might char with 75 per cent sulfuric acid, the urine was treated with lead acetate. The filtrate was freed from lead by adding 10 per cent H_2SO_4 and the final filtrate used in the Molisch test.

The absorption curves of the Molisch tests given by these biological fluids are shown in Figs. 1 and 2.

In accord with the recent report of Kozuka (2) we find that both human and rabbit blood contain ketoses.

Normal urine, as already shown by other workers, contains relatively large quantities of ketose.

The spinal fluids examined are of interest. In the normal fluid the absorption spectrum of a ketose is prominent. In the fatal case of meningococcic meningitis the ketose peak is almost absent and the aldose band is prominent, while during recovery the picture is intermediate between the normal and the fatal case. Other examinations show that at a later stage of recovery the fluid gives a Molisch test still nearer to the normal.

Normal saliva gives the spectrum of an aldose sugar only.

These observations confirm the report of Tashiro and Tietz (3) and add to it merely because the Molisch test as here used is more sensitive than their simple ketose test. Their test has a lower limit of sensitivity of about 0.03 per cent of levulose. For purposes of comparison the figures obtained by the Molisch test and those of Tashiro and Tietz are listed in Table III. It should be mentioned that the data from the Molisch test are tentative only. The error in estimation is about ± 4 per cent. This error has been reduced. As already mentioned, the results of the two tests differ only because the Molisch method is much the more sensitive.

SUMMARY

1. An improved α -naphthol test for sugars is described.
2. In this test aldoses give red pigments having an absorption maximum between 4800 Å. and 5100 Å. while ketoses give red-violet or blue-violet pigments with an absorption maximum between 5500 Å. and 5800 Å. Furfuraldehyde gives a red-violet Molisch test with maximum absorption between 5400 Å. and 5500 Å.
3. With this test, ketoses have been detected in normal and diabetic blood, normal urine, and normal and pathological spinal fluids. In meningococcic meningitis there is relatively less ketose

and more aldose in the spinal fluid than is found in normal fluid. During recovery from this disease there is a slow return to the normal Molisch test.

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THE ACTION OF EPINEPHRINE AND INSULIN IN FROGS UNDER ANAEROBIC CONDITIONS

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The predominantly oxidative metabolism of a homothermal animal represents such a complex system that it is usually difficult to decide what is the primary effect of a hormone in the body and what are merely secondary changes. For instance, the decrease in muscle glycogen and increase in blood lactic acid after epinephrine injection could be ascribed to a direct accelerating effect on the glycogenolytic process, but it could also be due to vasoconstriction and hence to a diminished supply of oxygen. Since it was found that epinephrine acts on muscle glycogen when supplied at a rate which causes dilatation rather than constriction of the blood vessels of muscle, the second possibility seemed less probable (1).

A further investigation of this problem is made possible by the fact that cold blooded animals are able to survive a period of oxygen want of several hours' duration. During anaerobiosis oxidative processes are eliminated or greatly restricted and the action of epinephrine and insulin, if any, can thus be studied under simplified conditions. Moreover, a possible curtailment of the oxygen supply to the tissues through vasoconstriction cannot be a factor in the action of epinephrine under anaerobic conditions. If, therefore, epinephrine should still be able to increase lactic acid formation when oxygen is excluded, this would have to be ascribed to a direct point of attack of epinephrine in the muscle cell.

Lesser (2-4) made numerous experiments concerning the effect of anaerobiosis on the carbohydrate content of frogs. He found that there occurred a progressive increase in sugar and lactic acid and that the former was derived from liver glycogen and the latter from muscle glycogen. Meyerhof and Meier (5) measured the rate of lactic acid formation in frogs kept anaerobically. In the

present experiments determinations of the sugar and lactic acid content of the whole animal were made in preference to glycogen determinations in liver and muscle, because the basal values of the former were less subject to individual variations than the latter. Lesser states that when glycogen is determined it is necessary to analyze at least fifteen frogs in each series in order to obtain a satisfactory average; according to our experience six to eight experiments are sufficient in the case of sugar and lactic acid determinations. Experiments on hepatectomized frogs to be described in the following paper (6) show that an increase in sugar content after epinephrine injection is dependent on glycogenolysis in the liver since it does not occur in the absence of the liver and that muscle glycogen is not a source of sugar but yields lactic acid. There is therefore some ground for the assumption that whenever the sugar or lactic acid content increases, this is due to a corresponding decrease in liver or muscle glycogen.

EXPERIMENTAL

Male frogs (*Rana pipiens*) of 20 to 35 gm. in weight were used. Since the experiments were made in the early winter months, the animals were in a good nutritional condition. They were kept in an aquarium at a temperature of about 10°. On the night previous to the experiment some frogs were placed in a cage standing in shallow water so that the animals could not submerge. For the experiments the frogs were put into large fruit jars provided with a rubber stopper, thermometer, and inlet and outlet tubes. The jars, which were kept in a water bath, had an inside temperature of $15 \pm 0.5^\circ$. Air or nitrogen, saturated with moisture, was passed through at a rate of about 15 liters per hour. In order to produce rapid anaerobiosis, one jar was filled with water and the nitrogen allowed to displace it. Traces of oxygen were removed from the nitrogen by passing the latter over copper gauze heated in a silica tube and then cooling it in a copper coil. When the nitrogen so prepared was bubbled for 20 minutes through 10 per cent pyrogalllic acid, only a slight yellow color was produced on addition of an equal volume of 10 per cent sodium hydroxide; *i.e.*, the Hopkins test for oxygen was negative.

After 3 hours the frogs were put into chamois bags containing CO₂ snow to which a few drops of ether had been added. When

thoroughly frozen they were cut into thin slices by means of a tobacco cutter and the slices placed in 50 cc. of ice-cold, 2 per cent sulfuric acid. The whole mass was frozen solid by addition of CO_2 snow and then allowed to thaw slowly at room temperature, which required about 30 minutes. During this time the acid had thoroughly penetrated into the tissues and passing them once through a small meat grinder sufficed to reduce them to a fine pulp. The tissue residue was separated on a Buchner funnel and was suspended three times in succession in 40 cc. of water, being filtered each time. The combined extracts were made up to 200 cc. and proteins were precipitated with mercuric sulfate, as described by West, Scharles, and Peterson (7). To 25 cc. of the extract, 20 cc. of water and 5 cc. of 30 per cent HgSO_4 in 10 per cent H_2SO_4 were added. Freshly precipitated barium carbonate was then added and the whole shaken until no more CO_2 was evolved and the reaction was faintly alkaline to neutral litmus paper. After filtration on a Buchner funnel and addition of a drop of strong sulfuric acid to precipitate the excess barium, H_2S was passed through the solution. The mercuric sulfide was filtered off and the H_2S expelled by aeration. After neutralization with NaOH aliquot portions were analyzed for sugar, the Hagedorn-Jensen and in some later experiments the Benedict method (8) being used. Lactic acid was determined according to Friedemann and Kendall (9) after removal of sugar by copper sulfate and lime.

Injections were made into the throat lymph sack. Epinephrine was given in doses of 0.005 to 0.1 mg. but in the majority of cases a dose of 0.02 mg. was used. Insulin (2 units) was injected 18 to 42 hours previous to the experiments. Control animals were given an equivalent amount of salt solution.

Recovery of Added Glucose and Lactic Acid—Amounts of glucose and lactic acid, covering the extreme ranges encountered in the present work, were added to the frog extract before precipitation with mercuric sulfate. The results obtained are shown in Table I. On an average 99.1 per cent of added glucose and 81.6 per cent of added lactic acid were recovered. Since the recovery of lactic acid in pure solution is between 95 and 100 per cent, it may be concluded that the mercuric sulfate precipitation leads to a loss of lactic acid amounting to nearly 20 per cent. A correction for this loss was not applied. It should be noted that other protein precipitants lead

to similar losses of lactic acid and besides, most of them do not yield a filtrate which is suitable for sugar determinations owing to the presence of a large amount of non-sugar reducing substances. The chief advantage of the mercuric sulfate precipitation is that sugar and lactic acid can be determined in the same filtrate and that the loss of the latter, though fairly large, is reasonably constant.

Fermentation with Yeast.—In order to determine the nature of the reducing substances in the filtrate from the mercuric sulfate precipitation, the filtrate was treated with washed yeast for 20

TABLE I

Effect of Mercuric Sulfate Precipitation on Recovery of Added Glucose and Lactic Acid

25 cc. of the same frog extract were precipitated in each case as described in the text.

Precipitation No.	Lactic acid				Glucose			
	Found	Added	Recovered		Found	Added	Recovered	
	mg.	mg.	mg.	per cent	mg.	mg.	mg.	per cent
1	0.312				0.129			
2	0.586	0.36	0.274	76	0.243	0.12	0.114	95
3	0.704	0.48	0.392	81.5	0.289	0.16	0.160	100
4	0.780	0.60	0.478	80	0.327	0.20	0.198	99
5	0.919	0.72	0.607	84.5	0.186*	0.12	0.122	101
6	1.085	0.90	0.773	86	0.215*	0.15	0.151	100.5
Average.....				81.6				99.1

* One-half of the usual amount taken for analysis.

minutes at room temperature. After centrifuging off the yeast, the supernatant fluid was used to make up a 0.01 per cent glucose standard from a 0.1 per cent glucose stock solution. At the same time 0.01 per cent glucose standards, the same pipette, stock solution, and volumetric flask being used, were prepared with the supernatant fluid of yeast that had been suspended in water and of yeast that had acted on a pure glucose solution. With the Benedict copper method, the three glucose standards gave the same reading within the limit of error of the method. This experiment was repeated three times on different filtrates and essentially the same result was obtained. It shows that the reducing substance

determined in the mercuric sulfate filtrate by means of the Benedict method is completely fermentable. When the Hagedorn-Jensen method was used, the glucose standard prepared with the fermented mercuric sulfate filtrate always gave higher reducing values than the other two standards. The non-fermentable residue in the case of the Hagedorn-Jensen method was found to be chiefly creatine which reduces the ferricyanide reagent even in the presence of glucose. Benedict's copper reagent is not affected by creatine in the presence of glucose, though creatine alone has a weak reducing action. Apparently the Benedict method gives true sugar values when applied to the mercuric sulfate filtrate, while the values obtained with the Hagedorn-Jensen method are somewhat too high. The difference in reducing value obtained by means of the Benedict and Hagedorn-Jensen methods on unfermented mercuric sulfate filtrates corresponds closely to the non-fermentable residue found in the latter method. This is illustrated in the following example. With the Benedict method 58.6 and with the Hagedorn-Jensen method 75 mg. of sugar per 100 gm. of frog were found, the difference being 16.4 mg. The non-fermentable reducing substance for the Benedict method was 0, for the Hagedorn-Jensen method 15.2 mg. per 100 gm. of frog. Generally the difference between the two methods was smaller than in the above example.

Incomplete Precipitation of Creatine—According to our present knowledge the most important reducing substances which interfere with the determination of the true sugar content of an acid extract of muscle are hexosephosphate, glutathione, and creatine. This will be discussed in a subsequent paper on muscle sugar. The former two substances are carried down by the mercuric sulfate-barium carbonate treatment of the extract. Creatine is, however, incompletely precipitated under these conditions. The concentration of creatine in the final filtrate used for the sugar analysis is remarkably constant, in spite of variations in its concentration before precipitation. The following figures per 2 cc. of final filtrate (this being the amount usually taken for sugar analysis) illustrate this point: 0.111, 0.108, 0.107, 0.098, 0.092, 0.105, 0.094, and 0.112, average 0.103 mg. of creatine¹ or 60 mg. of creatine per 100 gm.

¹The values recorded are those obtained with the alkaline picrate method after conversion of creatine into creatinine. The values obtained by means of the diacetyl color reaction for creatine were approximately 15 per cent lower.

of frog. Since creatine in the presence of glucose has a reducing power for the ferricyanide reagent approximately $\frac{1}{10}$ of that of glucose, 60 mg. of creatine increase the reducing value by about 6 mg. per 100 gm. of frog.

Comparison of the Hagedorn-Jensen and Benedict Methods—Before realizing the advantage which a specific copper reagent offers

TABLE II

Effect of Epinephrine Injections on Sugar and Lactic Acid Content of Normal and Insulinized Frogs under Aerobic and Anaerobic Conditions

The temperature was 15°. All values were calculated in mg. per 100 gm. of body weight.

	Aerobic (3 hrs.)				Anaerobic (3 hrs.)			
	Controls		Epinephrine-injected		Controls		Epinephrine-injected	
	Sugar	Lactic acid	Sugar	Lactic acid	Sugar	Lactic acid	Sugar	Lactic acid
Normal frogs	28.8	42.8	53.0	68.5	60.4	89.5	72.9	121.5
	33.6	42.5	49.0	63.0	65.0	96.5	82.0	134.0
	41.0	56.6	60.6	78.5	52.9	86.5	57.7	113.5
	40.3	49.0	59.0	62.0	74.0	82.4	70.1	97.5
	42.0	25.0	50.1	47.0	55.0	113.0	64.0	129.0
	41.7	35.6	53.1	51.6	56.5	121.1	64.9	166.0
	39.2	66.5	63.0	76.9	70.0	111.0	75.0	143.0
	35.4	60.0	52.5	65.0	41.0	90.5	71.0	131.0
	37.8	47.7	55.0	64.1	59.4	98.8	69.7	129.4
	±3.9	±10.9	±4.4	±7.4	±8.0	±12.3	±5.0	±14.3
	36.0	45.3	34.6	48.3	54.5	108.0	33.9	79.0
	26.9	54.1	33.9	56.5	46.5	81.1	56.0	81.5
	34.8	52.0	44.1	70.5	40.3	95.0	48.5	92.0
	39.5	57.0	42.5	57.5	35.8	71.5	42.0	102.0
All frogs injected with insulin 18 to 42 hrs. previously	31.6	40.1	38.2	53.1	50.0	86.5	50.5	100.1
	38.2	48.1	43.8	55.4	44.9	100.0	51.0	105.0
	34.5	49.4	39.5	56.9	45.3	90.3	46.9	93.2
	±3.5	±4.9	±3.8	±4.7	±5.3	±9.0	±6.0	±7.2

for this type of work, many determinations were made with the Hagedorn-Jensen method only. Recently comparisons between the two methods were made on thirty-seven filtrates from mercuric sulfate precipitations (Table III of this and Table III of the follow-

ing paper (6)). On an average the Hagedorn-Jensen method indicated 9.7 mg. more reducing substances per 100 gm. of frog than the Benedict method. In the preceding section it was shown that 6 out of the 9.7 mg. may be ascribed to the reducing action of creatine on the ferricyanide reagent. The nature of the remaining 3.7 mg. of non-sugar reducing substances was not ascertained.

*Effect of Epinephrine under Aerobic and Anaerobic Conditions—*In Table II two series of experiments are recorded, one aerobic and the other anaerobic. Each of these series is again subdivided into a control group and into a group injected with epinephrine. Whenever possible, experiments on four frogs, one for each of the four groups, were run on the same day. If this could not be done, experiments on one control and one injected frog of the same series were run at the same time. This is of some importance in the case of frogs, because experiments performed on the same day show much better agreement than experiments performed on different days. In order to examine individual experiments, figures on the same horizontal line should be compared. Epinephrine acting under aerobic conditions causes an increase in the sugar and lactic acid content of the frogs. This may be observed in each of the individual experiments. On an average the sugar content increased by 45.5 and the lactic acid content by 34.4 per cent. The response of the cold blooded animal to epinephrine is therefore of the same nature as that of the mammal.

When the frogs were kept for 3 hours in nitrogen, there resulted a marked increase in the sugar and lactic acid content, the former rising 57 and the latter 107 per cent. It should be noted that all frogs survived a period of anaerobiosis of 3 hours' duration, though some animals became paralyzed toward the end of the period. When placed in air, they recovered completely within a short time. Epinephrine injected at the beginning of anaerobiosis caused an increase in the rate of lactic acid formation. Each of the individual experiments was decidedly positive. The effect of epinephrine on the sugar content under anaerobic conditions was not nearly so marked as under aerobic conditions. Apart from a good deal of overlapping of the values, there was one negative (the fourth experiment in Table II) and several weakly positive experiments. On an average the increase in sugar content amounted to 17.3 per cent. It is probably correct to assume that epinephrine

has but a slight accelerating effect on sugar formation under anaerobic conditions.

The above experiments are open to the objection that there is oxygen left in the frogs when they are placed in nitrogen. At the rate of oxygen consumption prevailing at 15° it may roughly be calculated that 20 minutes would elapse until most of the oxygen is used up. During this period of partial anaerobiosis epinephrine might have exerted its influence on sugar and lactic acid formation. In order to test the validity of this objection experiments of the

TABLE III

Effect of Epinephrine Injections on Sugar and Lactic Acid Content of Decerebrated Frogs under Anaerobic Conditions

In these experiments (in contrast to those in Table II) the animals were analyzed 2½ hours after the beginning of anaerobiosis and 2 hours after the injection of epinephrine. All values were calculated in mg. per 100 gm. of body weight.

Controls			Epinephrine-injected		
Sugar		Lactic acid	Sugar		Lactic acid
Hagedorn-Jensen method	Benedict method		Hagedorn-Jensen method	Benedict method	
63.4		102.0	62.0		102.5
60.3		93.4	60.5		111.5
60.6	53.5	72.5	75.1	58.6	99.1
58.5	54.0	91.0	75.8	68.5	117.0
43.8	38.8	64.0	48.0	38.2	91.8
74.5	56.0	104.0	77.5	64.5	120.5
91.5	78.4	123.5	86.5	69.0	148.0
64.6	56.1	92.9	69.3	59.7	112.9

type shown in Table II were repeated 1 year later with the following modifications. The frogs were decerebrated by means of a Goltz puncture on the day preceding the experiment. As is well known, decerebrated frogs, when undisturbed, remain perfectly motionless for long periods of time. A possible error due to unequal muscular activity of the different animals was thus eliminated. Secondly, the animals were kept in nitrogen for a preliminary period of 30 minutes before epinephrine was injected. In this manner the action of epinephrine was confined to a period of

more complete anaerobiosis. Finally, the animals were kept under anaerobic conditions for a total period of only $2\frac{1}{2}$ hours. Owing to this fact they showed no signs of paralysis when removed from the containers. The results of this series of experiments are shown in Table III; on comparing them with the corresponding ones in Table II attention should be paid to the fact that epinephrine was allowed to act for a different length of time in the two cases. When calculated per hour, the excess lactic acid formation as the result of the epinephrine injection was the same; *i.e.*, an average of 10.0 mg. for the experiments in Table III and of 10.2 mg. for those in Table II. The conclusion that epinephrine is able to accelerate lactic acid formation under anaerobic conditions is therefore sustained.

The influence of epinephrine on sugar formation was even weaker in the experiments in Table III than in those in Table II. Glycogen determinations in the liver of decerebrated frogs showed that there was enough material present for sugar formation. There is the possibility that the anaerobiosis itself caused sugar formation to proceed near its maximal rate so that a further acceleration could not occur when epinephrine was injected.

Effect of Insulin under Aerobic and Anaerobic Conditions—In contrast to epinephrine which acts rapidly in cold as well as in warm blooded animals, insulin action has a marked temperature coefficient. The effect of temperature on the production of convulsions in frogs by insulin administration was studied in detail by Huxley and Fulton (10). They found that at 6–8° it took 120 to 144, at 15° 60 to 70, at 20° 43 to 49, at 25° 24 to 27, and at 30° 14 hours until hypoglycemic convulsions supervened. On plotting the rate of action of insulin and the O₂ consumption of frogs at different temperatures they noted a surprising coincidence between the two curves and by extrapolation they arrived at the conclusion that if a frog could exist for any length of time at 37°, convulsions would occur as rapidly as in a mammal.

In the present experiments the animals were used 18 to 42 hours after the insulin injection. Since they were kept at about 10°, they were still far removed from the convulsive state. Epinephrine, when injected into these insulinized frogs, produced hardly any increase in the sugar content either aerobically or anaerobically. But not only the glycogenolytic action of epinephrine was

antagonized by the preceding insulin treatment. The increase in sugar content which occurred when frogs were kept anaerobically was also markedly inhibited by the insulin injection (Table II). In mammals insulin prevented the decrease in liver glycogen which occurred at a certain time interval after the injection of epinephrine (11, 12).

Aerobic lactic acid formation after epinephrine injection and lactic acid formation produced by anaerobiosis were only slightly retarded by insulin administration, the former by 11 and the latter by 9 per cent. In rabbits insulin inhibited the rise in blood lactic acid after epinephrine injection by about 14 per cent (13). In view of these results it was surprising to find that the accelerating effect of epinephrine on lactic acid formation in anaerobic frogs was almost completely inhibited by insulin (Table II).

DISCUSSION

Lesser (3) determined the amounts of glycogen which disappeared from liver and rest of body during 3 hours of anaerobiosis at 14°. Per 100 gm. of frog an average of 112 mg. was lost from the liver and of 165 mg. from the rest of the body. In other experiments Lesser (4) determined sugar and lactic acid in the whole frog instead of glycogen. During 3 hours of anaerobiosis there were formed per 100 gm. of frog 102 mg. of sugar and 98 mg. of lactic acid. Meyerhof and Meier (5) found considerably less lactic acid formation during anaerobiosis, *i.e.* an average of 45 mg. per 100 gm. of frog in 3 hours. The value of 51 mg. found in the experiments in Table II agrees much better with that found by Meyerhof than with the value recorded by Lesser.

As stated in the introduction, the fact that epinephrine is able to accelerate lactic acid formation under anaerobic conditions makes it very improbable that this effect is due to vasoconstriction in muscle. The same conclusion was arrived at in experiments on mammals in which epinephrine raised the blood lactic acid (and blood sugar) level at rates of intravenous injection far below those required to raise the blood pressure. It is of interest that the amount of lactic acid formed as the result of epinephrine injection is nearly twice as large under anaerobic as aerobic conditions. The significance of this finding will be discussed in the following paper (6).

The idea that epinephrine is oxidized in the tissues and that it is this oxidized form in the nascent state which produces the effects, is not contradicted by the fact that epinephrine was found to act under anaerobic conditions. Traces of oxygen will undoubtedly remain in the tissues for some time in spite of anaerobiosis.

Von Issekutz (14) found that the isolated and perfused liver of frogs which had received an insulin injection on the preceding day, formed only one-fifth as much sugar as the liver of uninjected frogs. Furthermore, addition of epinephrine to the perfusion fluid increased sugar production much less in the liver of the insulinized frogs than in those without insulin injection. The present experiments on intact frogs may be regarded as a confirmation of these results; *i.e.*, an injection of insulin, 18 to 42 hours previously, prevented the glycogenolytic action of epinephrine on the liver. Insulin was also found to inhibit the hydrolysis of liver glycogen which takes place when frogs are kept for 3 hours in an atmosphere of nitrogen, showing that this action persists under anaerobic conditions. The inhibitory action of insulin on hepatic glycogenolysis has also been demonstrated in mammals; it possibly is one of the primary effects of insulin in the body.

SUMMARY

Epinephrine injections increased the sugar and lactic acid content of frogs under aerobic as well as anaerobic conditions. When epinephrine was injected into frogs which had received insulin 18 to 42 hours previously, an increase in sugar content did not take place either aerobically or anaerobically. This effect of insulin is ascribed to an inhibitory action on hepatic glycogenolysis, becoming especially noticeable when the latter is augmented by epinephrine or anaerobiosis. The fact that epinephrine is able to accelerate lactic acid formation under anaerobic conditions argues against the idea that this effect is due to vasoconstriction and hence to asphyxia in muscle. The evidence is in favor of the assumption that epinephrine has a direct accelerating effect on the glycogenolytic process in muscle.

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THE CALORIGENIC ACTION OF EPINEPHRINE IN FROGS BEFORE AND AFTER HEPATECTOMY

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Meyerhof (1) has shown that increased lactic acid formation in muscle is accompanied by an increase in respiration. This is due to the fact that part of the lactic acid formed is reconverted to glycogen under expenditure of oxidative energy. As Meyerhof found, for each oxygen equivalent of 1 mol of lactic acid used above the basal oxygen consumption, 2 to 5 mols of lactic acid may be reconverted to glycogen. Since epinephrine causes an increased lactic acid formation in muscle and an increase in oxygen consumption, it was natural to think of a connection between the two and to ascribe the calorigenic action of epinephrine to the extra expenditure of energy required for the reversion process. The present paper and others which are to follow, may be regarded as an examination of this hypothesis.

Calculation of Extra Oxygen Consumption—If the above hypothesis is correct, epinephrine injections in frogs should cause less lactic acid to appear under aerobic than anaerobic conditions, because part of the lactic acid formed aerobically would be reconverted to glycogen. In Table II of the preceding paper (2) the excess lactic acid formed as the result of epinephrine injection amounted to 306 mg. per kilo of frog under anaerobic and to 169 mg. under aerobic conditions, a difference of 137 mg. The extra oxygen consumption required for the removal of 137 mg. of lactic acid may be calculated as follows: Assuming a ratio (lactic acid disappeared: lactic acid oxidized) of 4:1, or of 137:34.2, one finds that 25.3 cc. of O_2 are required to oxidize 34.2 mg. of lactic acid. In other words, in the experiments in Table II of the preceding paper the frogs should have consumed an extra amount of 25.3 cc. of O_2 per kilo. The figure just arrived at offers a means of putting the above hypothesis to an experimental test.

Measurement of the Oxygen Consumption of Frogs—An apparatus similar to that described by Meyerhof and Meier (3) was used. A cylindrical flask of about 500 cc. capacity, provided with indentations for the support of a wire net, was connected by means of a ground joint with a Barcroft-Warburg manometer. 25 cc. of 10 per cent KOH were placed in the bottom of the flask for the absorption of CO_2 . The flasks were submerged in a large water bath provided with two stirrers and kept at a temperature of 15° . The calculation of the "vessel constant" was carried out as described by Warburg (4) and corrections for changes in temperature and barometric pressure were made by means of a thermobarometer. All values are expressed in cc. of O_2 per kilo of frog, reduced to standard conditions of temperature and pressure.

Immobilization of Frogs—In the present experiments it was necessary to measure the oxygen consumption for several consecutive hours, first under basal conditions and then after the epinephrine injection. Many fruitless attempts were made with normal and decerebrated frogs. The former remained under basal conditions for only short periods of time; the latter were sufficiently quiet when undisturbed, but the handling incidental to the injection caused them to move a good deal during the first half hour after the injection. It should be noted that the movements have a much stronger influence on the oxygen consumption of cold blooded than of warm blooded animals. Paralysis of the frogs by injection of drugs, such as curare, novocaine, or urethane, was undesirable for various reasons, and besides, it would have precluded the use of the same animals for experiments on several consecutive days.

The following procedure was finally adopted. The brain was divided by means of a Goltz puncture between the hemispheres and the talami optici. With a fine needle introduced into the spinal canal the cord was destroyed from the medulla downward. Such animals, when properly operated upon, are able to breathe spontaneously, but otherwise they are completely immobilized. They survive for several days and such operations as hepatectomy can be performed on them without the use of an anesthetic.

Plan of Experiments—The animals were immobilized on the day previous to the experiments. They were placed overnight in shallow water, care being taken to keep the nostrils free. It was found

advantageous to cover those parts of the body which remained outside of the water with wet gauze. In a number of experiments the liver was excised after all vascular connections were tied. On the following morning the animals were weighed, placed in the containers, and kept for several hours at 15°, so as to allow for adjustments of the metabolism to that temperature. Oxygen consumption was then measured for 2 consecutive hours. After 2 hours the frogs were removed from the containers, injected with 0.1 cc. of salt solution, and immediately replaced. This was done in order to test the effect of an injection on oxygen consumption. 10 minutes were allowed for the establishment of temperature equilibrium before the measurement of the oxygen consumption was resumed. After 1 hour the animals were again removed from their containers but this time they received an injection of 0.05 to 0.1 mg. of epinephrine into the throat lymph sack, otherwise the procedure was the same as after the injection of salt solution. The experiment was terminated 2 to 3 hours after the administration of epinephrine.

Effect of Epinephrine on Oxygen Consumption—In spite of the precautions taken, the oxygen consumption of frogs was not found to be as regular as that of mammals. This may be due to the fact that the frog as an aquatic animal is accustomed to periods of anaerobiosis and has therefore a metabolism less nicely regulated than that of mammals. The irregularities in O₂ consumption would undoubtedly disappear in observation periods of several hours duration, but unfortunately long metabolism periods were not practicable in the present work.

In the last two columns of Table I the increase in O₂ consumption after the epinephrine injection was calculated. In the majority of the cases epinephrine caused a rise in the O₂ consumption of the control as well as the hepatectomized animals and with few exceptions this rise was larger in the 1st than in the 2nd hour after the injection. During the 3rd hour after the injection the O₂ consumption had in all cases returned to the basal level.

The following discussion will be based mainly on the average values recorded in Table II. It may be seen that the O₂ consumption during the first and second basal periods of the control animals did not differ by more than 3.3 per cent; in the case of the hepatectomized animals the difference was even smaller. This indicates

TABLE I
Influence of Epinephrine on O₂ Consumption of Immobilized Frogs

	Frog No.	O ₂ consumed per kilo of frog per hr. at 15°						Increase in O ₂ over average basal consumption	
		Basal		After salt solution injection	After epinephrine injection				
		1st hr.	2nd hr.		1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.
		cc.	cc.	cc.	cc.	cc.	cc.	per cent	per cent
Controls, 1st day	2	36.5	33.3	32.4	44.9	48.9	30.8	31.7	43.4
	6	39.6	45.2	43.9	42.5	55.2		0.1	30.8
	8	30.2	25.8	36.4	54.1	39.0		75.5	26.6
	10	47.0	38.8	52.0*	58.1	54.5		35.5	21.3
	11	19.7	22.7	25.7	54.5	43.1	26.1	140.0	90.1
	12	25.7	18.3	18.3	25.8	33.2		24.0	59.6
	13	18.1	27.1		51.5	34.2	24.4	128.0	51.3
	14	27.8	37.6	45.9	57.2	43.0		54.1	15.9
Controls, 2nd day	11	34.7	34.7		55.0	33.8	32.5	58.5	-0.3
	12	33.7	35.4		66.3	65.6	37.1	92.1	84.4
	13	36.8	30.2	26.8	42.8	38.6		37.2	24.4
Hepatectomized, 1st day	7	43.2	35.7	41.7	53.1	44.2		31.7	10.0
	9	32.0	24.2	32.0	46.1	28.0		56.7	-0.5
	15	22.7	25.8	26.8	33.1	26.8		31.9	6.8
	16	29.1	24.9	21.8	41.5	29.1	26.6	64.6	15.5
	17	27.2	28.3	31.6	46.7	30.5	30.5	61.0	5.2
	18	12.2	26.6	26.6	37.1	33.8	26.0	70.1	55.0
Hepatectomized, 2nd day	7	26.9	31.9		47.0	37.8	27.4	59.9	28.6
	9	37.0	27.1	36.0	43.7	35.4		30.8	6.3
	17	25.8	29.3	29.3	37.0	26.4	26.9	31.7	-7.1
	18	22.0	25.7	23.6	26.8	30.1	23.6	13.1	27.0
Hepatectomized, 3rd day	9	33.7	28.5	33.4	35.0	28.5		9.7	-10.3
	17		28.0		36.0	30.1		28.6	7.5
	18	25.7	24.6		29.7	30.2		18.3	20.3
Hepatectomized, 4th day	17	26.5	26.5		38.6	29.0		45.6	9.4
	18	27.3	25.2		29.6	30.4		12.9	6.0

* This animal was able to make slight movements.

that the number of experiments was large enough to eliminate the irregularities in the O₂ consumption which were encountered in individual experiments.

The handling of the animals incidental to the injection of salt solution (or of water) produced a slight rise in the O_2 consumption, amounting to 8.8 per cent in the control and to 8.7 and 5.0 per cent in hepatectomized animals. The average basal O_2 consumption (inclusive of the metabolism period after the injection of salt solution) amounted to 33.2 cc. per kilo of frog per hour for the control animals. Deducting this from the O_2 consumption in the 1st and 2nd hours after the epinephrine injection one finds $50.2 - 33.2 = 17.0$ and $44.2 - 33.2 = 11.0$, a total of 28.0 cc. of extra O_2 consumption as the result of the epinephrine injection. This agrees

TABLE II

Influence of Epinephrine on O_2 Consumption of Immobilized Frogs (Average Values of Table I)

Type of experiment	No. of frogs used	O_2 consumed per kilo of frog per hr. at 15°					Increase in O_2 over average basal consumption	
		Basal		After salt solution injection	After epinephrine injection			
		1st hr.	2nd hr.		1st hr.	2nd hr.	1st hr.	2nd hr.
		cc.	cc.	cc.	cc.	cc.	per cent	per cent
Controls (liver intact)	11	32.8	31.7	35.1	50.2	44.2	51.2	33.1
1st day after hepatectomy.	6	27.7	27.5	30.0	42.9	32.0	51.0	12.7
2nd " " "	4	27.9	28.5	29.6	38.6	32.4	35.0	13.3
3rd " " "	3	25.7	26.3		32.8	30.1	26.4	15.8
4th " " "	2	26.9	25.8		33.7	29.7	28.2	12.9

very well with the value of 25.3 cc. which was arrived at by calculation in a preceding section (p. 367) and lends support to the conception that the calorogenic action of epinephrine is due to reconversion of lactic acid.

On the 1st day after removal of the liver the extra oxygen consumption after the epinephrine injection amounted to 18.1 cc. as compared to 28.0 cc. for frogs with intact liver, showing that the calorogenic action of epinephrine is diminished after hepatectomy. It may be assumed that part of the calorogenic action takes place in the liver, since a reconversion of lactic acid to glycogen has been demonstrated in that organ following epinephrine injection

(5). Apart from the removal of the liver, the response to epinephrine depends on the condition of the animals. On the 2nd and 3rd days after hepatectomy (plus destruction of most of the cen-

TABLE III
Effect of Epinephrine Injections on Sugar and Lactic Acid Content of Immobilized Frogs

The temperature was 15°. The animals were analyzed 2 hours after the injection and all values were calculated in mg. per 100 gm. of body weight.

	Controls			Epinephrine-injected		
	Sugar		Lactic acid	Sugar		Lactic acid
	Hagedorn-Jensen method	Benedict method		Hagedorn-Jensen method	Benedict method	
Liver intact	40.0	33.0	39.8	74.0	65.4	58.6
	63.5	59.9	59.4	63.2	59.9	64.5
	55.6	41.2	33.6	57.0	45.0	48.0
	58.5	56.6	54.0	92.6	89.0	81.6
	59.0	57.0	67.1	63.5	55.2	75.0
	59.1	53.5	60.0	67.0	65.0	86.1
	57.0	42.5	52.1	84.1	62.1	65.5
	56.1	49.1	52.2	71.6	63.0	68.4
	±4.7	±8.0	±7.8	±9.2	±8.2	±8.3
	61.5	48.5	61.4	54.3	41.0	78.0
	72.5	55.0	54.0	62.5	42.9	65.7
	46.0	36.1	45.5	44.0	24.0	59.0
Hepatectomized	62.5	53.2	53.0			61.4
	41.5	28.0	69.0	58.5	43.5	72.0
	37.6	20.2	52.3	40.5	23.2	56.6
	57.4	45.5	58.1	60.1	58.5	67.5
	57.2	41.6	78.6	45.6	31.8	95.0
	43.6	36.7	49.8	38.1	34.6	74.2
	26.0*	21.7*	41.4*	50.0	36.4	74.3
	53.3	40.5	57.9	50.5	37.3	70.4
	±9.5	±9.3	±7.8	±7.6	±7.3	±8.3

* 3 days after hepatectomy; not included in average.

tral nervous system) as the condition of the animals declined, the calorigenic action of epinephrine became progressively weaker and in some individual experiments (Frog 9 on the 3rd day and Frog 18

on the 4th day, Table I) it disappeared entirely. Since weak calorogenic actions were also observed in pithed frogs with intact liver when the animals were in poor condition, there is reason to believe that the lack of response to epinephrine some time after hepatectomy is due to the approaching death of the animal.

Sugar and Lactic Acid Formation in Immobilized and Hepatectomized Frogs—The average lactic acid content of the immobilized frogs in Table III was practically the same as that of normal frogs (52.2 as compared to 47.2 mg. per 100 gm.). The former animals (like those used for the metabolism experiments in Table I) were immobilized 24 hours prior to the experiment. Since the lactic acid content did not rise appreciably during that time, the O_2 consumption after immobilization must have remained large enough to prevent an accumulation of lactic acid.

An increase in the sugar and lactic acid content was observed when epinephrine was injected into immobilized frogs with intact liver (Table III). In contrast to this epinephrine failed to cause a rise in the sugar content of immobilized and hepatectomized frogs. As in the case of the mammal (Mann (6), Soskin (7)), sugar production in frogs is a function of the liver and in the absence of this organ there is apparently no other carbohydrate reserve present which can yield glucose. Though the muscles of these hepatectomized frogs contained considerable amounts of glycogen, the latter was not changed to glucose when epinephrine was injected. There was, however, a rise in lactic acid following the injection of epinephrine into hepatectomized frogs.

DISCUSSION

The calorogenic action of epinephrine which is ascribed to the reconversion of lactic acid in liver and muscle is in some respects similar to the specific dynamic action of amino acids. According to our present knowledge the increased oxygen consumption following the administration of amino acids represents the energy required for the conversion of amino acids to carbohydrate and in the case of alanine the intermediary products on the path of this transformation would actually be either lactic or pyruvic acid. Since the catabolism of amino acids depends on their deamination in the liver (Bollman, Mann, and Magath (8)), it seems probable that this organ is the principal site of extra heat production after

amino acid ingestion. Similarly, in mammals the lactic acid which escapes from muscle during epinephrine action is reconverted to glycogen in the liver under expenditure of oxidative energy.

The calorigenic action of epinephrine, though somewhat diminished in intensity, persists after removal of the liver in the frog. The question as to whether epinephrine acts calorigenically in mammals after removal of the liver has not been decided with certainty. Soskin's (9) experiments cannot be accepted as final, because they were performed on animals with a rapidly declining oxygen consumption. His conclusion that epinephrine injections did not arrest this rapid decline should not be interpreted to mean that epinephrine might not raise the oxygen consumption under more favorable experimental conditions. The observation made in experiments on frogs, that the calorigenic action often disappeared a few hours before death, lends support to this interpretation.

There is reason to believe that the O_2 consumption of normal resting muscle (as that of active muscle) is connected with the glycogen \rightleftharpoons lactic acid cycle described by Meyerhof. Some glycogen is constantly converted to lactic acid and the latter is removed at the expense of oxidative energy. Epinephrine by accelerating the breakdown of glycogen to lactic acid in muscle would thus raise the heat production by an intensification of normal muscle metabolism. Justification for this conception is seen in the fact that the efficiency of the reversion process during epinephrine action, as measured by the ratio (total lactic acid disappeared: lactic acid oxidized), was found to be of the same order of magnitude as in normal muscle, *i.e.* 4 to 1. Meyerhof and Meier (3), using intact frogs, found a value for the above ratio of 5 to 1 during rest and of 4.5 to 1 during recovery from muscular activity. A further discussion of the calorigenic action of epinephrine requires a consideration of the results obtained on mammals and will be presented later.

SUMMARY

Following the injection of epinephrine into immobilized frogs, the O_2 consumption rose on an average 51 per cent in the 1st and 33 per cent in the 2nd hour with a return to the basal level in the 3rd hour. The total extra O_2 used during this period was 28 cc. per kilo of frog, while the extra O_2 calculated from the difference in

aerobic and anaerobic lactic acid formation on the basis of a 4 to 1 reconversion ratio amounted to 25.3 cc. In immobilized and hepatectomized frogs the extra O_2 consumption after epinephrine injection was 18 cc. per kilo or 35 per cent less than in immobilized frogs with intact liver. On the 2nd and 3rd days after removal of the liver, as the condition of the animals declined, the calorogenic action of epinephrine became progressively weaker. The sugar content of hepatectomized frogs did not rise when epinephrine was injected; there was, however, an increase in the lactic acid content. It is assumed that the calorogenic action of epinephrine in frogs is due to an accelerated lactic acid formation with subsequent reconversion of this lactic acid in muscle and liver, a process which is known to be accompanied by expenditure of oxidative energy.

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STUDIES ON THE INACTIVATION OF CATALASE

III. DESTRUCTION OF CATALASE BY HYDROGEN PEROXIDE

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Experimental studies of the catalase reaction which have been made within recent years, have resulted in the recognition of the fact that this consists of two independent processes: (1) the enzymatic decomposition of hydrogen peroxide by catalase with the liberation of molecular oxygen; and (2) the destruction of catalase. The application of the usual equations for expressing the reaction velocity of catalase has met with little success, the different formulas proposed having fitted more or less some particular set of experiments only. Williams (1) suggests that the second part of the process is not due to a direct action of the hydrogen peroxide upon the enzyme but rather to the action of the nascent oxygen as it is set free. Unfortunately this hypothesis cannot be tested experimentally. However, Michaelis and Pechstein (2) have actually demonstrated that the reaction is unaffected, whether or not the oxygen is removed or allowed to accumulate, and Northrop (3), from his analysis of the results of my earlier catalase determinations, has shown that the decrease of the enzyme was a function of time and not a function of the oxygen produced. Maximowitsch and Awtonomowa (4) have developed an elaborate equation describing the catalase reaction in which the two separate processes are taken into consideration. In previous studies, we (5) found that the second process is markedly affected by temperature, so that at 2° where the destruction of the catalase is least, the enzymatic action attains a maximum. Furthermore, it was found that the second process is greatly dependent upon the

hydrogen peroxide concentration as well as temperature. We found repeatedly and without exception that under identical experimental conditions increasing the hydrogen peroxide concentration resulted always in a lowering of the oxygen liberated (*i.e.* of the enzymatic process). Indeed, by making the hydrogen peroxide concentration high enough the catalase reaction could be practically stopped. Inasmuch as all our experiments were made with fairly high concentrations of hydrogen peroxide, and realizing that such a condition is not generally favorable for the study of the reaction kinetics, we repeated the experiments with smaller concentrations. Owing to the fact that small amounts of oxygen had to be measured, we could not employ our usual gasometric method, but resorted instead to the titration of aliquots with KMnO_4 , using both 0.1 and 0.01 *N* solutions, depending upon the magnitude of the titration. Samples were withdrawn at definite intervals from the reaction mixture and transferred to a flask containing sulfuric acid. The reaction was thus brought at once to a standstill, and the titration was carried out as usual. Repeating our earlier experiments, but under the condition of much greater dilution, we were surprised to find that, instead of the decrease in catalase activity, which we invariably observed to follow every increase in the hydrogen peroxide concentration, the activity was directly proportional to the catalase concentration and *independent* of the peroxide concentration. This deviation from our previous experience was so striking that it called for careful analysis, the results of which form the subject of the present note. Inasmuch as we decreased only the catalase and peroxide concentrations of the system, leaving everything else unchanged, it occurred to us that possibly the difference in results was not primarily due to the lower concentration of hydrogen peroxide employed but to the relatively higher concentration of the buffer used to maintain a constant reaction throughout the experiment. We therefore considered first just how much buffer (Sørensen's phosphate mixture) was required to secure constancy of results. These experiments were carried out with the same catalase preparation and under essentially the same conditions as before (5); namely, at 21°, at a pH of about 7.0, and a total volume of the reaction mixture of 50 cc. Inasmuch as we adjust the reaction of the different reagents to a pH 7.0, little buffer is required to maintain the reaction

of the medium practically constant and to secure uniformity of results. It is not surprising, therefore, that with a definite concentration of catalase the same amount of oxygen was set free from a given concentration of hydrogen peroxide, even up to 0.108 N, while the buffer concentration of the system was varied from as high a concentration as 0.07 M to as low as 0.00001 M. But although the catalase reaction was thus found to be independent of the buffer concentration within a very wide range, the situation

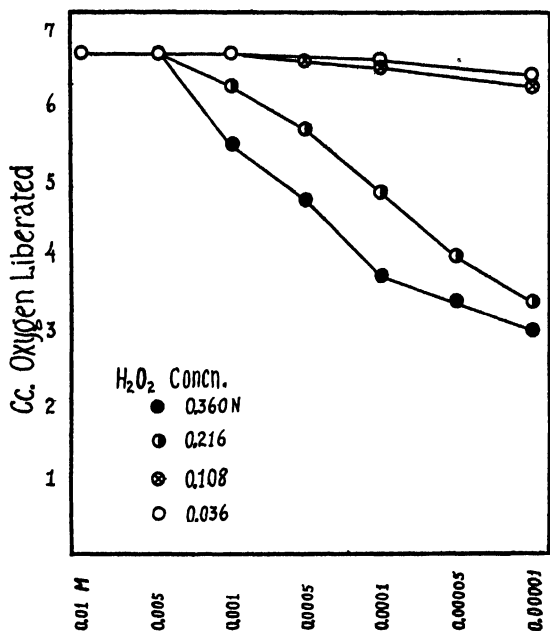


FIG. 1

was quite different when the hydrogen peroxide concentration was raised in the presence of varying amounts of the buffer mixture. This can best be seen by referring to the curves obtained by plotting the results of a number of determinations. In Fig. 1 it can be seen that with a buffer concentration varying from 0.01 to 0.00001 M there is little variation in the catalase activity when the hydrogen peroxide concentrations are below 0.1 N. With higher peroxide concentrations there is a clearly recognizable tendency

to a diminution in the oxygen liberated when the buffer concentration has been very greatly reduced. This is apparent with 0.108 N peroxide and becomes much more extensive and appears even at a much smaller diminution in the buffer concentration as the hydrogen peroxide is increased to 0.216 and 0.360 N. The thought, of

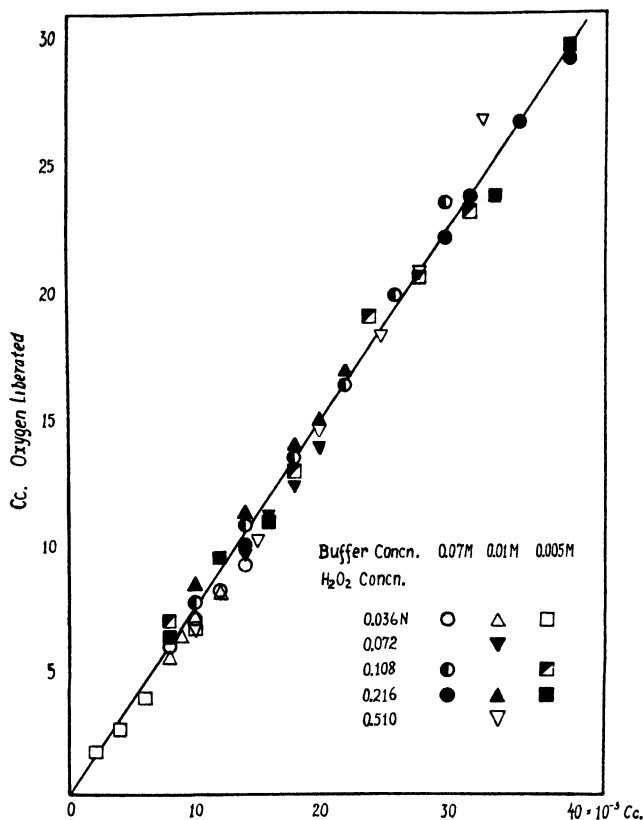


FIG. 2. Oxygen liberated plotted against cc. of catalase solution

course, occurs immediately that this diminished catalase activity may be due to a lowering of the salt concentration of the system and not at all to the inefficient buffering effect. But when sodium chloride is added in sufficient amount to give the same final salt concentration in all the experiments the results are identical with

those shown in Fig. 1. In other words, the difference in catalase activity with increasing concentrations of hydrogen peroxide is really a function of the degree of buffering of the system. It will also be observed that even at a 0.005 M buffer concentration the catalase activity is unaffected by the changes in hydrogen peroxide.

In Fig. 2, the catalase activity with hydrogen peroxide ranging from 0.036 to 0.510 N is shown in mixtures of a buffer concentration varying from 0.005 to 0.07 M, and it is obvious that the activity is a linear function of the catalase concentration. In other words, under these conditions of the experiment the catalase activity is

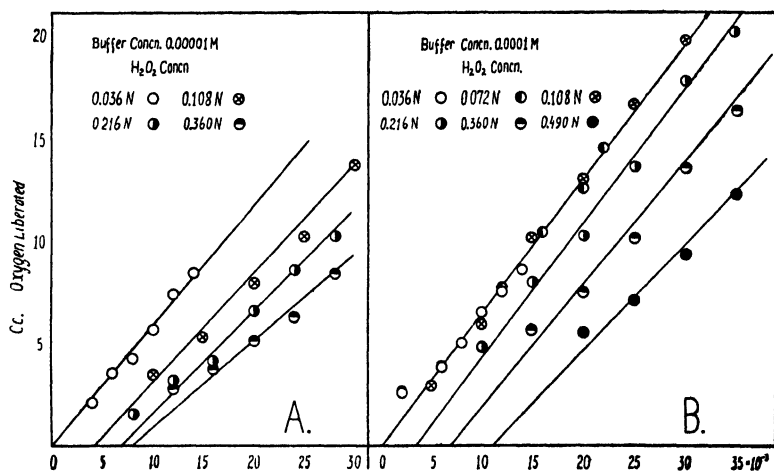


FIG. 3. Oxygen liberated plotted against cc. of catalase solution

directly proportional to the enzyme concentration and is independent of the peroxide concentration. If the catalase functioned as a catalyst we could not expect such a result, since the end result of the reaction should be independent of the concentration of the catalyst, while here we find exactly the opposite.

If the experiments are performed with a much smaller concentration of buffer, the reaction is still proportional to the catalase concentration but is no longer independent of the peroxide concentration, as can be seen from an examination of the curves in Fig. 3. Considering Fig. 3,B first, it will be observed that with the 0.0001 M buffer concentration the enzyme activity is directly proportional

to its concentration and is still independent of the peroxide concentration within the range of 0.036 to 0.108 *N* hydrogen peroxide. However, as the peroxide concentration is being increased further to 0.216 *N* and more, the catalase activity becomes progressively less. But the depressing effect of an increasing peroxide concentration is fully manifested in the experiments, where the buffer concentration had been reduced to only 0.00001 *M*. In this case, as can be seen from Fig. 3,A, the catalase activity is affected even by a small increase in the peroxide concentration so that the oxygen set free by the same amount of catalase becomes definitely dependent upon the concentration of the substrate. It is also to be noted that, when the catalase reaction is so depressed, the direct proportionality between the catalase and the amount of peroxide undergoing decomposition is less perfect and more limited as the concentration of hydrogen peroxide increases. These experiments thus indicate that the destruction of catalase by hydrogen peroxide is affected not only by temperature but even to a greater extent by the degree of buffering of the reaction system. In the presence of sufficient buffering the catalase reaction becomes strictly proportional to the concentration of the enzyme and independent of the concentration of the substrate (hydrogen peroxide), but as the buffering becomes less efficient the catalase activity becomes more and more dependent upon the hydrogen peroxide concentration.

It is necessary to point out here that the concentrations of buffer employed in these experiments have only relative significance, and the concentration which in this series was still sufficient might be insufficient in other experiments where more catalase and peroxide are being employed. However, a concentration of even 0.01 *M* seems to furnish sufficient buffering under conditions of wide variation in the concentration of the hydrogen peroxide to secure proportional results.

SUMMARY

The destruction of catalase by hydrogen peroxide which was found to increase with the temperature has now also been shown to depend even more markedly upon the degree of buffering. The effect is not due to a low salt concentration, as it can be reproduced even when the salt concentration is kept constant.

In the presence of sufficient buffering the catalase reaction be-

comes directly proportional to the concentration of the enzyme and independent of the hydrogen peroxide concentration.

If the buffering is insufficient, the catalase reaction decreases with increasing hydrogen peroxide concentration and, furthermore, with greater concentration of peroxide the proportionality between enzyme and oxygen set free also tends to become less general.

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THE EFFECT OF INGESTED COTTONSEED OIL ON THE COMPOSITION OF BODY FAT*†

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It has been shown in earlier work (1) that cottonseed oil differed from the other plant oils studied in its hardening effect on the body fat of hogs. When fed to the extent of 4 per cent of the ration, corn, peanut, and soy bean oils had a distinct softening effect, whereas cottonseed oil had the opposite effect. Abnormally high values for the melting point and for the saturated acid content of the hog fat were the chief characteristics noted for the cottonseed oil.

A similar effect has been found in connection with the feeding of cottonseed meal. Harrington and Adriance (2) and, later, Hare (3) showed that the lard produced by hogs fed a ration of corn and cottonseed meal was firm and had a high melting point. In the dairy industry, the production of tallowy butter through the excessive feeding of cottonseed meal has also been noted. The work of Eckles and Palmer (4) showed that the presence of either cottonseed meal or added oil in the ration affected the composition of the butter. Their data indicated that the volatile

* This work was conducted as a part of the soft pork project now included in the national project, "Cooperative meat investigations." The authors are indebted to Mr. E. Z. Russell for his interest in providing the hogs and facilities for the feeding experiments and to Mr. J. H. Zeller for supervision of the feeding experiments and compilation of the growth and feeding data. They are also indebted to Mr. E. W. Sheets and Dr. Paul E. Howe for their interest and cooperation in the conduct of the work.

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‡ Resigned.

and soluble acids normally present in butter were displaced in part by acids of higher molecular weight, presumably derived from the cottonseed oil.

Recently, Anderson and Mendel (5) have published data showing that the rat when fed a ration containing 60 per cent of the total calories in the form of cottonseed oil deposited fat with approximately the same iodine number as the ingested oil. The results for other oils such as peanut, soy bean, and corn were similar.

One of the problems in the study of soft pork has been to find suitable hardening feeds available for use in conjunction with the softening feeds. Cottonseed meal is a valuable protein supplement for use with corn. Because of difficulties encountered when it was fed to hogs, the use of this feed was restricted for many years. Lately, interest in this field has been revived and recent experiments indicate that cottonseed meal may come into more general use as a hog feed. That it has hardening rather than softening properties adds to its value.

The present investigations have been restricted to a study of the effect of cottonseed oil on the character of body fat. Particular attention has been given to the effect of different levels of intake of the oil on the firmness and composition of the body fat of the hog. As the feeding experiments progressed, it became evident that an unusual transformation in the composition of the fat was taking place. The investigation was accordingly extended with the view of seeking the explanation for the metabolic process involved.

Hog Feeding Experiments

In a preliminary experiment, corn, peanut, and cottonseed oils were added to a basal ration of barley and tankage. The oil content of the mixed ration was 3 per cent. At this level, the peanut and corn oils did not cause a significant increase in the softness of the hog carcasses, which were only barely distinguishable from those of the cottonseed oil-fed lot. However, the melting point of the fat of the lot fed cottonseed oil was 36.7°, as compared to values of 31.3° and lower for the other lots.

Corn and tankage were used as the basal feeds in the next experiment. The oils were incorporated at a 4 per cent level. In

addition to the three oils named, soy bean oil was included in this series. A fifth lot of hogs was fed at a later date a mixture of corn and tannage with 11.5 per cent of added corn oil. Results of these tests given in a previous article (1) are presented in Table I for comparison with the subsequent experiments.

In the case of peanut, soy bean, and corn oils, it was known that increasing quantities of these oils either naturally contained in or added to hog rations result in increasingly soft carcasses. The effect of 4 per cent of cottonseed oil, as already noted, raised the question of the possible effects of higher levels of oil intake. It did not appear reasonable to expect proportionately greater firm-

TABLE I
Effect of Added Oils on the Firmness of the Carcass and Composition of the Lard

Ration	Firmness grade*	Fatty acid separation			
		Iodine No.	Oleic	Linoleic	Total saturated
			per cent	per cent	per cent
Corn and tannage + 4.1 per cent peanut oil.....	MS	72.4	47.9	13.8	32.5
" " " + 4.1 " " soy bean oil...	MS	75.7	43.3	18.6	33.8
" " " + 4.1 " " cottonseed oil.	H	64.4	35.9	15.7	43.0
" " " + 4 1 " " corn oil.....	MS	76.3	45.0	16.8	33.0
" " " + 11.5 " " " ".....	O	97.2	41.4	31.4	23.1

* The grades are H, hard; MS, medium soft; O, oily.

ness of the body fat from increasing quantities of the oil in the feed. From the work of Jamieson and Baughman (6, 7) on the composition of vegetable oils, data are available for comparison of the fatty acid composition of the oils already mentioned together with rice oil and others of interest in hog feeding. All are high in oleic and linoleic acids. The actual percentages of the several fatty acids, although they show significant differences for the various oils, offer nothing of contrast between cottonseed oil and the other oils to suggest any difference in their utilization in the formation of fat.

An experiment was accordingly planned in which cottonseed

oil was fed at 4, 8, and 12 per cent levels. Two basal rations were chosen. One, which contained less than 1 per cent of naturally contained fat, was composed of hominy, tankage, alfalfa meal, and mineral mixture.¹ The other ration was composed of ground yellow corn, tankage, and a mineral mixture.¹ Seven lots of three hogs each and one lot of four hogs were fed in the usual manner for periods of approximately 100 days. A check lot on the different

TABLE II
Growth and Feed Consumption

Item	Basal feed, corn				Basal feed, hominy			
	Basal ration only	Basal ration with cottonseed oil			Basal ration only	Basal ration with cottonseed oil		
		4 per cent	8 per cent	12 per cent		4 per cent	8 per cent	12 per cent
No. of hogs	3	3	3	3	3	3	3	4
Average duration of test, <i>days</i>	96	107	96	66	107	107	96	98
Average initial weight, <i>lbs</i>	76	62	67	75	63	69	69	72
Average final weight, <i>lbs</i>	212	204	203	194	185	214	201	207
Average daily gain per hog, <i>lbs</i>	1.42	1.32	1.42	1.80	1.14	1.35	1.37	1.38
Feed consumed per 100 <i>lbs</i> . gain, <i>lbs</i> ..	372	328	327	366	335	284	271	332
Estimated consumption of oil (naturally contained), <i>lbs</i>	21.9	19.5	18.3	16.7	5.1	5.6	5.2	6.9
Added cottonseed, <i>lbs</i>		18.3	35.3	52.4		16.3	28 0	53.8

levels of oil was fed on each of the two basal rations. The hominy ration was considered especially desirable, because of its low fat content, to demonstrate the effect of the cottonseed oil alone. The corn ration with approximately 4 per cent of naturally contained oil had been used in the previous experiment. The feeding results are shown in Table II. No difficulties due to either the toxic

¹ The mineral mixture consisted of 10 parts of wood ashes, 10 parts of 16 per cent acid phosphate, and 1 part of common salt.

TABLE III
Firmness Grading and Fat Constants

Lot No.	Hog No.	Slaughter weight	Firmness grade*	Average thickness, back fat†	Refractive index at 40°		Back fat	
					Back fat	Leaf fat	Iodine No.	Melting point
Series A; basal feed, corn								
I, control	1	205	H	36	1.4596	1.4586	62.9	40.2
	2	209	MH	35	1.4596	1.4584	63.8	40.8
	3	197	H	41	1.4595	1.4587	60.4	40.6
Average.....		204		39	1.4596	1.4586	62.4	40.5
II, 4 per cent oil	4	204	H	39	1.4598	1.4587	65.9	48.0
	5	188	H	40	1.4600	1.4591	64.6	48.2
	6	194	H	38	1.4596	1.4586	63.3	48.4
Average.....		195		39	1.4598	1.4588	64.6	48.2
III, 8 per cent oil	7	184	MH	43	1.4608	1.4595	74.1	46.4
	8	201	MH	35	1.4611	1.4598	72.9	45.4
	9	186	H	54	1.4600	1.4592	67.8	47.3
Average.....		190		44	1.4606	1.4595	71.6	46.4
IV, 12 per cent oil	10	167	S	37	1.4620	1.4611	82.3	40.5
	11	172	S	45	1.4620	1.4605	83.2	37.3
	12	220	S	46	1.4622	1.4609	85.4	36.9
Average.....		186		49	1.4621	1.4608	83.6	38.2
Series B; basal feed, hominy								
V, control	13	195	MH	35	1.4589	1.4580	60.7	35.6
	14	174	H	28	1.4592	1.4584	59.7	40.7
	15	166	H	37	1.4592	1.4586	60.2	38.5
Average.....		178		33	1.4591	1.4583	60.2	38.3
VI, 4 per cent oil	16	217	H	35	1.4597	1.4583	61.4	48.4
	17	195	H	32	1.4590	1.4580	56.8	48.0
	18	212	H	43	1.4598	1.4588	62.6	48.2
Average.....		208		37	1.4595	1.4584	60.3	48.2

* The grades are H, hard; MH, medium hard; MS, medium soft; S, soft; O, oily.

† Based on average of five measurements of the back fat.

TABLE III—*Concluded*

Lot No.	Hog No.	Slaughter weight	Firmness grade*	Average thickness, back fat†	Refractive index at 40°		Back fat	
					Back fat	Leaf fat	Iodine No.	Melting point
Series B; basal feed, hominy— <i>Concluded</i>								
VII, 8 per cent oil	19	lbs. 192	H	mm. 32	1.4604	1.4591	69.2	47.0
	20	182	H	36	1.4595	1.4582	62.5	48.2
	21	195	H	39	1.4597	1.4587	63.6	47.5
	Average.....		190		36	1.4599	1.4587	65.1
VIII, 12 per cent oil	22	195	MS	26	1.4617		81.7	37.0
	23	201	MH	40	1.4602		68.5	41.4
	24	201	MS	40	1.4610		75.2	39.0
	25	201	MS	39	1.4607		72.5	38.2
Average.....		200		36	1.4609		74.5	38.9

principle (gossypol) of cottonseed or other causes were encountered in the feeding of the highest levels of oil.

The gains of the individual hogs were uniformly satisfactory. The data on feed consumption per 100 pounds of gain indicate unusually good utilization of the feed. The estimated consumption of oil, divided between that naturally contained which was calculated from the ether extract and the added cottonseed oil, is given in Table II. The total body fat deposited by the hogs during the feeding period can be roughly estimated at 50 to 60 pounds. Thus sufficient oil was ingested on the 12 per cent levels of both rations to account for the quantity of fat deposited.

The hogs were slaughtered at the close of the feeding periods. According to the usual practice in these and similar investigations, the carcasses were graded for firmness. As shown in Table III, the maximum firmness of the hogs fed corn was reached in Lot II, fed 4 per cent of oil. Increase in the oil content of the ration fed Lots III and IV to 8 and 12 per cent respectively led to increasing softness. All three carcasses of Lot IV were graded soft. However, they were by no means as soft as the group in a previous experiment (Table I) fed a corn ration with 11.5 per cent of added corn oil. The gradings on the carcasses in Series B indicate

harder carcasses than in Series A. All those in Lots VI and VII were graded hard while in Lot VIII, three were graded medium soft and one medium hard. The refractive index and iodine number values increase in each series. Thus the control lots show the lowest fat constants although two of the six carcasses were graded medium hard. The hominy-fed lots show consistently lower fat constants than the corn-fed lots.

This can be explained by the difference in the oil content of the basal rations. Lot VII on hominy with 8 per cent of oil is approximately the equivalent of Lot II on corn with 4 per cent of oil, with the other lots in corresponding order. Lot IV, fed 12 per cent of oil, was by far the softest. Melting point determinations follow the order of firmness rather than that of the iodine number and refractive index. Outside of the high values found on samples from cottonseed oil feeding, few melting point values over 40° have been found among the many determinations made on lards in this laboratory. Even on the fat samples taken from extremely hard carcasses of hogs fed on brewers' rice, the maximum value noted was 42.7; the iodine number on this particular sample was 52.0. It is evident that the cottonseed oil was responsible for the abnormal rise in melting points just as it caused the rise in iodine and refractive index values. The maximum firmness of the carcasses as well as melting point of the fat was reached in the lots which received 4 per cent of oil. The fraction of the fat which raised the melting point to a maximum in these lots was either replaced or partially hidden by increasing quantities of an unsaturated fraction responsible for the rise in iodine and refractive index values.

After the individual samples had been analyzed, composite samples of back fat were prepared on each lot. Iodine numbers, melting points, and saponification numbers were determined on these samples. The results are given in Table IV. The average refractive indexes taken from Table III are included for comparison. The iodine numbers and melting points on the combined samples agree in the main with the averages given in Table III. The saponification numbers do not show any significant changes.

The lead salt-ether separation into saturated and unsaturated fractions was next made on the eight composite samples. After correction for the saturated acids carried into the unsaturated

fraction and for certain minor losses due to analysis, the per cent of oleic and linoleic acids as well as of total saturated acids contained in the fat was calculated. In two cases, namely Lots VI and VII, the unsaturated acids were brominated and then separated according to the well known procedure involving differences in solubility in petroleum ether and benzene. The bromine content of the fractions was next determined. The results on the benzene-insoluble portion showed 0.09 and 0.12 per cent of the total fat for Lots VI and VII respectively to be arachidonic acid. These values are within the range obtained in this laboratory on a

TABLE IV
Composition of the Body Fats

Lot No.	Average refractive index at 40°		Composite back fat			Fatty acid separation		
	Leaf fat	Back fat	Iodine No.	Melting point	Saponification No.	Oleic	Linoleic	Total saturated
				°C.		per cent	per cent	per cent
Series A								
I	1.4586	1.4596	62.8	38.2	196.3	50.4	8.7	36.3
II	1.4588	1.4598	64.0	46.2	196.3	34.5	16.4	44.5
III	1.4595	1.4606	71.0	44.2	196.3	34.5	19.8	41.4
IV	1.4608	1.4621	83.8	35.5	195.6	32.3	28.2	34.9
Series B								
V	1.4583	1.4591	60.6	41.7	196.2	47.9	8.5	39.0
VI	1.4584	1.4595	60.5	45.7	197.9	38.2	12.7	45.5
VII	1.4587	1.4599	64.4	46.3	197.6	34.2	17.4	44.0
VIII		1.4609	77.4	40.9	195.2	30.4	25.4	39.6

wide variety of lards. The results of the lead salt-ether separation shown in Table IV disclose some striking changes in composition. It will be noted that the oleic acid declined and the linoleic acid increased in each series until in the lots given 12 per cent oil the latter was within 5 per cent of the content of the former. These changes account for the increases in iodine numbers and refractive index values already noted. Just as the firmness grades and the melting points reached maximum values on the lots given 4 per cent oil, so, too, does the saturated acid content. The figures for Lots II and VI do not differ greatly from the value given in Table I which was obtained in the earlier experiment.

Lots III, IV, VII, and VIII show a decrease in total saturated acid per cent although the figures for the lots given 12 per cent oil were similar to those on the control lots even though there was a wide difference in the firmness of the carcasses. The formation and deposition of these high proportions of saturated acids raised the question of the relative amounts of the several saturated acids normally present in fats. Previous work (8) has shown that palmitic acid usually predominates in hog fat. Indeed, it may constitute two-thirds of the total saturated acids. From the melting points of the fats, the presence of an abnormally high proportion of stearic or a saturated acid of even higher molecular weight with high melting point was suspected.

Further analysis of the saturated acid fraction of Series B was next undertaken. Suitable quantities of this fraction were pre-

TABLE V
Distribution of Saturated Acids

Lot No.	Total saturated acids in fat	Myristic acid	Palmitic acid	Stearic acid
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Series B				
V	39.0	1.6	24.3	13.0
VI	45.5	1.1	24.1	20.3
VII	44.0	0.8	20.9	22.3
VIII	39.6	1.0	13.2	25.3

pared from the composite samples of Lots V to VIII. Following the esterification of the acids, the esters were fractionated *in vacuo*.

Following the necessary analyses on these fractions, the fatty acids were reclaimed from the residues, purified by repeated crystallizations from alcohol, and melting points determined. Stearic and palmitic acids were easily identified in the fractions on which the mean molecular weights indicated them to be present. Melting points of 69.0–70.5° for stearic acid, 62.0–63.5° for palmitic acid, and 58–59° for myristic acid were obtained. With these confirmatory tests completed, the content in terms of the whole fat was next calculated for the three acids. The results are shown in Table V.

The distribution of the three acids in Lot V, the control lot, is similar to that found in earlier investigations on samples produced on a variety of rations. The actual values are typical of a normal, firm lard produced on rations with such basal feeds as brewers' rice, hominy, corn, and other feeds low in ether extract. Lots VI to VIII show increasing quantities of stearic acid with a decrease in palmitic acid. The proportions of the two acids in Lot VIII are the reverse of those in Lot V. Although the analysis of cottonseed oil (6) shows 20 per cent palmitic acid and only 2 per cent stearic, there was actually a displacement in the body fat of the palmitic acid with the stearic acid.

Early in this investigation, the question was raised regarding the isomeric forms of the fatty acids deposited in the body fat. The possibility of the presence of isooleic acid in the fat was considered in view of the high iodine numbers as well as high melting points. The complete analysis of the fat with identification and determination of the fatty acids has eliminated the possibility of isooleic acid as an important constituent in the body fat. It is evident that the stearic acid was formed in unusually large quantities in the animal body under the conditions of the experimental feeding. In view of the high content of unsaturated acids in the oil as well as the quantity of oil ingested, the source of the stearic acid would appear to be in this fraction. The analyses of the body fats show a decrease in the oleic acid along with an increase in linoleic acid which suggests the former acid as the source. In other words it may be reasoned that one or more isomeric forms of oleic acid are present in cottonseed oil which are readily converted into stearic acid by saturation in the animal body.

At this point it may be well to point out the bearing of these results in hog feeding. Cottonseed meal contains from 5 to 10 per cent of oil. When fed at the rate of 1 part in 10 of a ration, the cottonseed oil content, with an average of 8 per cent in the meal, would be only 0.8 per cent. However, where the meal is fed at the rate of 1 part in 4 or 5, the oil content would approach 2 per cent. This quantity may not have any pronounced influence, nevertheless it would be a favorable one even though limited.

Effect of Cottonseed Oil on Body Fat of Rats

During the time the work with hogs was in progress, feeding experiments with rats were undertaken with the purpose of deter-

mining the effects of cottonseed oil and products derived from it on the composition of the body fat of rats. At the outset it was realized that the rate of fat formation per unit of body weight was low in the rat as compared to that in the hog. This difference renders comparison of results a difficult one and no doubt has a bearing on the results obtained. The basal ration consisted of hominy or brewers' rice 63 parts, skim milk powder 30 parts, alfalfa meal 5 parts (in several cases 10 parts), monocalcium phosphate 1 part, and sodium chloride 1 part. The desired amount of oil or fat was added to this ration by substitution of an equivalent amount of the hominy or brewers' rice. Cottonseed oil was fed at 3, 6, 9, and 12 per cent levels in one series for comparison with the hog feeding experiment.

In view of the results which had been obtained on the hog fat, it appeared desirable to test with rats whether the hardening properties resided in the saturated or in the unsaturated fractions. Sufficiently large portions of these fractions were prepared by the lead salt-ether method for feeding purposes. The fractions were incorporated at a 6 per cent level in the first trial. Later a second series was run with only 3 per cent added to the basal ration.

Crisco, the partially hydrogenated vegetable oil product, was used in another series to determine whether the stearic acid presumably formed by hydrogenation would affect the composition of the body fat.

Albino rats ranging in weight from 75 to 125 gm. were placed on the experimental rations. Male rats were used for the most part since they appeared to store the greatest quantity of fat in a given period. The feeding periods varied from 46 to 100 days. When the animals reached approximately 200 gm. in weight, they were chloroformed and placed in a refrigerator. The fat was carefully rendered after which refractive index, iodine number, and melting point determinations were made.

During the progress of these experiments the results of the carefully planned and controlled experiments of Anderson and Mendel became available (5). In their comparison of a wide variety of oils and fats, they noted that when soy bean, corn, cottonseed, or peanut oils were fed, the resulting body fat resembled the food fat. However, their diet contained 60 per cent of the total calories as fat which amounted to 37.2 per cent by

weight. In view of the results from hog feeding experiments in this as well as earlier work, it is not surprising to find the close resemblance of food fat and body fat even in the case of cottonseed oil. The present work with rats (Table VI) deals with a much lower oil intake. The results on the cottonseed oil series show a marked increase in softness of the fat to the maximum fed of 12

TABLE VI
Effect of Cottonseed Oil on Body Fat of Rats

Item	No. of rats	Days on experiment	Average gain	Refractive index at 40°	Iodine No.	Melting point	Iodine No. of ingested fat or oil
			gm.			°C.	
Basal rations.....	7	100	97	1.4591	59.1	31.0	
Cottonseed oil, 3 per cent.....	4	66	71	1.4599	62.7	29.4	108.5
“ “ 6 “ “	7	70	104	1.4612	75.9	31.4	108.5
“ “ 9 “ “	4	46	79	1.4619	80.5	30.6	108.5
“ “ 12 “ “	4	51	121	1.4630	94.2	25.8	108.5
Crisco, 3 per cent.....	3	56	67	1.4598	63.3	28.5	71
“ 6 “ “	4	45	74	1.4603	68.5	30.3	71
“ 12 “ “	4	51	142	1.4607	75.0	29.7	71
Cottonseed oil saturated acids, 3 per cent	2	46	90	1.4598	58.7	32.7	20.0
Cottonseed oil saturated acids, 6 per cent.....	2	80	89	1.4587	51.0	27.2	15.0
Cottonseed oil unsaturated acids, 3 per cent.....	4	55	103	1.4612	75.2	29.4	144.5
Cottonseed oil unsaturated acids, 6 per cent.....	2	91	83	1.4611		30.0	137.0

per cent. At this level, the iodine number of the body fat was 94.2. The use of Crisco caused an increase in the iodine number and refractive index. A 12 per cent intake resulted in a more unsaturated body fat than the ingested Crisco. Results of feeding the fatty acid fractions show the production of relatively firm fat on the saturated fraction and the opposite on the unsaturated fraction. However, the resulting body fat in neither case reached

the extremes of the ingested fractions. Possibly selective absorption together with difficulty of absorption of the high melting point constituents of the saturated fraction may explain the apparent desaturation in the body fat of the rats fed the saturated acids. Similarly the actual procedure involved in the apparent saturation of the ingested unsaturated fraction can only be conjectured. Whether the oil or the fractions had the same effect in the rat as the oil did in the hog on the fatty acid distribution was not possible to determine. Neither was it possible to grade the fatty tissues of the rats for firmness. However, the melting point values did not decrease in order with increases in iodine numbers. Indeed in the oil-fed series, the 6 per cent intake yielded the maximum melting point which was only exceeded by one group fed the saturated fraction of the oil. The feeding of Crisco did not cause a change in the melting point. It is of interest to note that the values for the groups fed the unsaturated fraction are little different from those of other groups. This fact suggests that a certain amount of stearic acid may have been formed from the unsaturated fraction by the rat, as had occurred in the hog, as explanation of the maintenance of melting point in the face of increasing refractive index and iodine number. Although there are suggestions of possible similarities in behavior of the oil in the rat and the hog in the present experiments, the results in the former are in general not sufficiently marked to be noteworthy outside of the apparent softening produced by increasing quantities of oil in the ration.

SUMMARY

Earlier work showed that the ingestion of cottonseed oil by hogs resulted in the formation of a firm body fat characterized by a high melting point and a high saturated acid content. In order to determine the level at which maximum firmness was produced, experiments were conducted on hogs in which the oil was added to basal rations at 4, 8, and 12 per cent levels. The hardest carcasses were secured on the 4 per cent level. The higher quantities resulted in increasing softness.

Analysis of the lard showed striking changes in the fatty acid distribution. Among these was a marked increase in the linoleic and the stearic acids at the expense of the oleic and palmitic acids.

Although the maximum content of total saturated acids occurred in the 4 per cent level, the maximum in stearic acid increased steadily up to the 12 per cent level. It is suggested that the changes in composition of the lard are due to the presence in cottonseed oil of one or more isomeric forms of oleic or possibly linoleic acid which are readily converted into stearic acid.

Cottonseed oil feeding tests on rats gave a more pronounced softening of the body fat than was obtained in hogs. There were indications that changes in the fat composition occurred in the rat as in the hog following ingestion of cottonseed oil.

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STUDIES ON THE SPONTANEOUS OXIDATION OF CYSTEINE

II. THE AUTOXIDATION OF CYSTEINE FREE FROM IRON*

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While there is now a general agreement that iron is an important catalyst in the spontaneous oxidation of cysteine itself and of the cysteine peptides such as glutathione, and it is certain that both iron and cysteine compounds exist in many if not all forms of living matter, it is still uncertain whether cysteine is capable of self-oxidation in the complete absence of iron. Thus, Warburg and Sakuma (1) maintain that spontaneous oxidation is impossible without iron, while Abderhalden and Wertheimer (2) believe that cysteine itself will oxidize at a slow rate provided some cystine be present. The question is of particular importance in interpreting the nature of the action of cyanides on living matter. These substances almost universally depress respiration. If all respiration depends upon iron, then cyanides may act (Warburg) by combining with iron to make a non-ionic form, thus inhibiting respiration. If, however, self-oxidation can take place in the absence of iron, then an explanation of the action of cyanides other than that suggested by Warburg must be looked for.

That certainly some self-oxidations do not depend upon iron is indicated not only by the unlikelihood of atmospheric oxygen combining with only a single element, iron, but also by the fact that while cyanides depress respiration, they do not entirely inhibit it.

The universal presence of iron in cells of all kinds, and its well

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known power of catalyzing a great variety of oxidations, clearly indicate its importance in cellular respiration. In view of the several conflicting theories which attempt to explain the oxidation of cysteine in terms of iron catalysis and the inhibiting action of cyanides in terms of the formation of an inactive cysteine-iron-cyanide complex, or the removal of a catalytically active cystine-cysteine complex, Professor J. U. Lloyd suggested a study of the action of iron in cell respiration. This investigation was accordingly carried out under the direction of Professor A. P. Mathews in accordance with Professor Lloyd's general suggestion.

EXPERIMENTAL

Method of Oxidation Measurement—The oxidation rates were determined by means of the blood gas microrespirometer of Barcroft and Roberts. Clove oil, colored with small amounts of Sudan III and having a specific gravity of 1.032, was used as the manometer fluid. This particular density is chosen so that 760 mm. of mercury corresponded to 10,000 mm. of clove oil. The reaction bulbs of the apparatus were submerged in a water bath which was maintained at a constant temperature. The total volume of each reaction system was 38.556 cc. The amount of oxygen absorbed during an experiment was calculated by the use of the following formulæ.

$$v(x - y) \quad 2r \left(\frac{V}{P} + S \right)$$

where

$v(x - y)$ = volume of oxygen absorbed

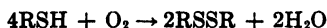
V = volume of air space in c.mm. of each reaction system, namely 38,556 c.mm.

$2r$ = difference in level of manometer fluid in mm.

S = cross sectional area of manometer tube, 1.566 sq.mm.

P = atmospheric pressure in mm. of clove oil

A 20 mg. sample of cysteine hydrochloride dissolved in 5 cc. of water and adjusted to the proper hydrogen ion concentration with purified alkali solution, in oxidizing completely to cystine according to the following equation,



will require under standard conditions 0.000,507 gm., or 710.3 c.mm. of oxygen. Under actual conditions of the experiment at a

temperature of 23° and a pressure of 749.6 mm. of mercury, the theoretical requirement is 780.2 c.mm. of oxygen. An experiment showed that after a period of continuous shaking for 100 hours, oxygen was no longer taken up by the solution and the difference in manometric level was 155 mm. Accordingly, the volume of O₂ absorbed was

$$\begin{aligned} v(x - y) &= 2r \left(\frac{V}{P} + S \right) \\ &= 155 \left(\frac{33,420.36^*}{9744.8} + 1.566 \right) \\ &= 774.23 \text{ c mm} \end{aligned}$$

* The volume of air space is equal to 38,556 less 5,000 c.mm. and 135.64 c mm. occupied by the cysteine solution and barometric liquid respectively.

This represents 99.3 per cent of the theoretical amount. This, with other tests, indicates that the sample of cysteine hydrochloride used in this and other experiments was extremely pure and contained little or no cystine.

Rate of Oxidation of Purified Cysteine—A series of experiments was carried out to determine the rate of oxidation of the pure, iron-free, cysteine hydrochloride prepared according to the method described in a previous paper (3). As had been already noted by other investigators, the rate of oxygen uptake when determined on equal amounts of the same sample of cysteine hydrochloride and when run under identical conditions varies quite widely although within definite limits. The cause or causes of this variability has been undetermined, but is most probably due to a slight variation in the pH of the solutions used, although they were adjusted as carefully as possible by colorimetric methods. That cysteine oxidation is very sensitive to the hydrogen ion concentration was shown by Mathews and Walker (4). Because of this slight variation, a number of determinations were usually carried out for each experiment and the average used in calculations.

The rate of shaking, regulated by a small electric motor attached to a shaking apparatus, was adjusted so as to give 190 to 210 complete oscillations per minute. In a previous experiment, it

had been found that although the rate of oxygen uptake when the system is at rest is considerably slower than when shaken, yet when the uptake is slow, as it is when no iron is present, it is independent of the shaking rate within rather wide limits. This may be explained by the fact that there is always present in the solution a sufficient amount of oxygen to satisfy the extremely slow rate of oxidation of iron-free cysteine. The limiting factors in the rate of absorption in this investigation are cysteine concentration, hydrogen ion concentration, and the amount of iron added, rather than the speed of solution of the oxygen. This may be shown by the fact that the velocity of oxygen uptake of pure cysteine is approximately linear over a considerable period of time and it is only near the very end of the oxidation, when the amount of unoxidized cysteine is considerably diminished, that the rate appreciably decreases.

This linear reaction rate in the first stages of oxidation of cysteine in the presence of a considerable amount of ionized iron may be explained by the possibility that a definite amount of the latter can bring about the oxidation of a definite amount of cysteine in a given time. Towards the end of the reaction the falling off in rate corresponds to the decrease in concentration of available cysteine below that corresponding to the iron present. The falling off in velocity with decreasing concentration of cysteine is, of course, modified by the autocatalytic nature of the reaction.

That the amount of oxygen present in the system is not a limiting factor may be shown by the following consideration. 20 mg. of cysteine hydrochloride under laboratory conditions will require, as has been explained previously, 780 c.mm. of O_2 , and since the 33,000 c.mm. of air held in the system, additional to the 5 cc. of cysteine solution, contain approximately 6600 c.mm. of O_2 , it is evident that there is present nearly 9 times as much oxygen as is actually required to convert the amount of cysteine used in an experiment into cystine.

Samples of the water and ammonia solutions used in these experiments were evaporated in 500 cc. quantities in quartz dishes, and the theoretical residues (no visible residue remained) tested for iron by the modified thiocyanate method (3) previously described. In no case was the slightest trace of color observed. This would indicate, from a consideration of the sensitivity of the

test by which one may detect 1 part of iron in 10 million parts of the substance in question, that the 5 cc. of ammonia solution, used in neutralizing the cysteine hydrochloride in the oxidation determinations, contain no appreciable amount of iron and certainly something less than 0.000,000,001 gm. of iron. When 2 gm. of the purified cysteine hydrochloride were ignited in a quartz crucible and tested for iron by the same method, a negative test resulted. Accordingly, 20 mg. of the cysteine hydrochloride contain certainly less than 0.000,000,001 gm. of iron. The maximum amount of iron which could possibly be present in the experiment as a contamination of the cysteine hydrochloride and of the solution used and still escape detection due to limits of sensitivity of the modified thiocyanate method must be something less than 0.000,000,002 gm. Any oxidation taking place, then, must be attributed either to this extremely small trace of iron which may be present but has not been shown to be, or to the autoxidizability of the cysteine itself.

The amount of alkali solution (NH_4OH iron-free) required to bring 20 mg. of cysteine hydrochloride to a pH of 7.6, determined by a separate titration, was added to the cysteine hydrochloride in the reaction bulb together with sufficient pure water to make the total volume 5 cc. To the compensatory bulb were added 5 cc. of ammonia solution having a pH of 7.6. The hydrogen ion concentration values were checked colorimetrically both before and after each determination.

The apparatus was then shaken with the stop-cocks open in the water bath for $\frac{1}{2}$ hour in order to allow the temperature of the solution in the bulbs to reach that of the bath. The shaking was then stopped and the apparatus brought to an exactly vertical position so that the manometer fluid reached the same level in both arms. The stop-cocks were then closed, shaking begun, and the barometric pressure recorded. Readings were taken every hour or two, or whenever convenient during runs of long duration. 3 minutes were allowed to pass after shaking periods for equilibrium to be reached before readings were taken.

The results of a typical experiment are recorded in Table I. In this experiment, 5 cc. of sodium hydroxide solution were used as the alkali to neutralize the cysteine hydrochloride and contained to the extent of about 0.0001 mg., which accounts for the

TABLE I

Oxidation of Cysteine Hydrochloride

20 mg. of cysteine hydrochloride in NaOH solution; pH, 7.4; temperature, 22.0–22.6°; barometric pressure, 749 mm. of Hg or 9737 mm. of clove oil; shaking rate, 180 oscillations per minute.

Time elapsed from beginning	Manometer tube readings		Difference in pressure (B – A)	Total O ₂ uptake
	A	B		
<i>hrs.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	<i>c.mm.</i>
0.0	124.0	124.0	0.0	0
4.0	121.0	127.0	6.0	30
8.0	116.5	131.5	15.0	75
23.0	102.0	146.0	44.0	220
29.5	95.0	153.0	58.0	290
45.0	80.0	168.0	88.0	440
56.0	69.0	179.0	110.0	551
58.5	66.5	181.5	115.0	576
69.5	57.5	190.5	133.0	666
73.0	55.7	192.3	136.5	683
80.5	50.5	197.5	147.0	736
93.0	47.0	201.0	154.0	771
100.0	46.5	201.5	155.0	776

TABLE II

Rate of O₂ Uptake of Pure Iron-Free Cysteine Hydrochloride Neutralized by Iron-Free Ammonia

Experiment No.	Time of run	pH	Temperature	O ₂ uptake per hr. per 10 mg. RSH·HCl
	<i>hrs.</i>		<i>°C.</i>	<i>c.mm.</i>
2	24.0	7.4	23.0–23.4	2.4
3	20.5	7.6	24.0–25.5	3.2
4	21.0	7.4	22.5	1.8
5	12.0	7.5	22.8–23.0	2.1
6	8.0	7.4	23.0	1.6
7	51.0	7.5	22.0–23.0	1.9
8	24.0	7.5	22.5–23.0	2.3
9	8.0	7.6	22.0–23.0	0.9
10	22.0	7.4	24.0–24.5	2.0
11	21.0	7.5	23.0	2.5
12	12.0	7.4	24.0	3.6

Average uptake = 2.21 c.mm. of O₂ per hour per 10 mg. of RSH·HCl.
Shaking rate = 180 to 200 oscillations per minute.

increased rate of oxidation as compared to those in which only pure ammonia solution was used. 100 cc. of the sodium hydroxide solution when tested in the manner described above contained about 0.002 mg. of Fe. In all of the remaining experiments ammonia solution only was used in adjusting hydrogen ion concentrations. Because of the great amount of time required for the oxidation of pure cysteine to go to completion and since the rate was linear over a period of 24 hours, experiments were interrupted after such a linear period of sufficient length was obtained. The results of a series of tests which illustrate the extremely slow rate of oxidation for pure cysteine are given in Table II. It will be noted that the oxidation rates are expressed in terms of oxygen uptake for 10 mg. samples of cysteine hydrochloride rather than for the 20 mg. samples actually used in the experiments. This is done to make the rates comparable with those expressed and calculated from data of other workers, which are given in Table III. It may be noted that when the uptake rates obtained by Sakuma (5) are recalculated in terms of oxygen uptake per hour per 10 mg. sample of cysteine hydrochloride, the average of the oxidation rates of his purest sample is 1.22 c.mm. of O_2 per hour, while that of Harrison (6), calculated in a similar manner, is 3.47 c.mm. of O_2 per hour. These values are of the same order as those obtained in this study. The variability of the oxidation rates, when oxidation is carried out under similar conditions, is evident in Sakuma's results where oxygen uptake rates as low as 0.47 c.mm. of O_2 per hour and as high as 3.25 c.mm. of O_2 per hour are recorded. Sakuma quotes no determinations for iron in his paper but from a consideration of the extremely slow oxidation rates for his cysteine, there is little doubt that his preparation was iron-free.

Harrison states that 0.1 gm. of his pure cysteine hydrochloride contained 0.0001 mg. of iron. Since the 2 gm. sample prepared in this laboratory failed to give a test for iron and since the maximum amount it could have possibly contained and still escape detection is 0.0001 mg. of iron, it must be concluded therefore that this purified cysteine hydrochloride is at least 20 times more pure than that of Harrison. This comparison is striking when one considers that the oxidation rate of this highly purified cysteine is practically the same as that of Harrison's sample in spite of the apparent greater degree of purity.

TABLE III

Rates of Oxidation of Cysteine as Obtained by Other Investigators

Preparations	Size of sample	pH	O ₂ uptake per hr.	O ₂ uptake per 10 mg. RSH·HCl	Investigator
	mg.		c.mm.	c.mm.	
Cysteine HCl	2000.0	7.6	7000.0	35.0	Mathews and Walker
" "	9.1	7.6	114.0	125.0	Dixon and Tunnicliffe
Crude RSH·HCl	10.0	8.0	380.0	380.0	Harrison
" " *	16.0	6.8	191.0	120.0	Sakuma
" " *	16.0	9.2	370.0	231.0	"
Impure RSH I*	16.0	6.8	13.9	8.7	"
" " "	16.0	7.6	15.6	9.8	"
Pure RSH II	24.8	7.7	3.66	1.47	"
" " "	42.3	7.7	3.35	0.79	"
" " "	16.3	7.7	0.77	0.47	"
" " "	66.8	7.7	21.70	3.25	"
" " "	22.6	7.7	6.10	2.30	"
" " "	24.8	7.7	3.7	1.5	"
" " "	42.3	7.7	3.4	0.8	"
" " A	10.0	7.7	6.22	6.22	Harrison
" " B	10.0	7.7	2.49	2.49	"
" " "	12.0	7.7	4.21	3.50	"
" " C	9.0	7.3	3.32	3.69	"
" " "	10.0	7.6	2.32	2.32	"
" " "	10.0	7.7	4.60	4.60	"
" " "	10.0	7.7	2.80	2.80	"
" " "	10.0	7.8	5.00	5.00	"
" " "	13.5	8.0	4.80	3.55	"
" " "	36.0	8.0	17.50	2.32	"
" " "	10.0	7.7	6.60	6.60	"
" " "	12.0	7.7	4.20	3.50	"
" " "	10.0	7.7	2.80	2.80	"
" " "	13.5	8.0	4.80	3.55	"
" " "	10.0	8.0	7.20	7.20	"

* Temperature in all cases was 20.0° except samples marked with * in which case the temperature was 37.5°.

Effect of Iron on Cysteine Oxidation

It was found by Sakuma that amounts of iron as small as 0.000,000,1 gm. produce a measurable action. His results are summarized in Table IV. All of the figures have been recal-

culated in terms of oxidation rates per hour per 10 mg. sample of cysteine HCl.

TABLE IV
Effect of Iron on Cysteine Oxidation (Sakuma)

O ₂ uptake per hr with no iron	Amount of iron added	O ₂ uptake per hr. after iron addition
c mm.	mg.	c mm.
3 25	0 00050	12 4
0 80	0 00020	6 0
2 30	0 00017	8 5
1 50	0 00010	4 5

Average = 1.96 c.mm. of O₂ without addition of iron. The results are calculated from the data in the original paper in terms of 10 mg. samples of cysteine hydrochloride.

If we assume from his data that 1.96 c.mm. of O₂ per hour is the oxidation rate of pure cysteine HCl, then

0.00050 mg. Fe increases rate 10.4 c.mm. O₂ (2.1 c.mm. O₂ per 0.00010 mg. Fe)
 0.00020 " " " " 4.0 " " (2.0 " " " 0.00010 " ")
 0.00017 " " " " 6.5 " " (3.8 " " " 0.00010 " ")
 0.00010 " " " " 2.5 " " (2.5 " " " 0.00010 " ")

From such a consideration it may be seen that the increase in oxygen uptake averages 2.6 c.mm. of O₂ per hour per 0.00010 mg. of Fe. The general average uptake per 10 mg. sample of cysteine hydrochloride free from iron per hour for all of the experiments reported in his paper in which he used his purified Sample II is 1.22 c.mm. of O₂.

Then, if, as the figures indicate, the increase in oxidation rate is proportional to the amount of iron added, and if we assume that the residual oxidation is due to the presence of iron in the sample, then from a simple algebraic ratio,

$$2.6 \text{ c.mm. O}_2 : 0.00010 \text{ mg. Fe} :: 1.22 \text{ c.mm. O}_2 : x \text{ mg. Fe}$$

$$x = 0.000,047 \text{ mg. Fe present}$$

the amount of iron present which we must assume to be responsible for this oxidation of the 10 mg. of cysteine hydrochloride is calculated to be 0.000,047 mg. of Fe, an amount which might easily have been detected on igniting a 1 gm. sample of cysteine hydro-

chloride and testing with KCNS solution. He does not cite any tests of his cysteine for iron.

Harrison's results, when calculated in terms of uptake per 10 mg. sample of cysteine HCl per hour, are summarized in the same manner. The value taken for the oxidation rate of his purest sample is the average rate expressed in Table III. The data for a number of his experiments appear in Table V.

TABLE V
Effect of Iron on Cysteine Oxidation (Harrison)

O ₂ uptake per hr. with no iron	Amounts of iron added	O ₂ uptake per hr. after iron addition
<i>c.mm.</i>	<i>mg.</i>	<i>c.mm.</i>
6 6	0.00010	15.60
3 5	0.00020	16.50
2 8	0.00010	12.80
	0.00160	101.00
	0.00120	75.00
	0.00080	54.00
	0.00040	29.00

Average rate when no iron is added = 3.47 c.mm. of O₂ per hour. See Table III of the original paper. The results are calculated, from data in the original paper, in terms of uptake per 10 mg. sample of cysteine hydrochloride.

In this case if we assume the oxidation rate of his pure cysteine HCl to be 3.47 c.mm. of O₂ per hour per 10 mg. of RSH·HCl then,

0.0016 mg. Fe increases rate 97.5 c.mm. O₂ (6.1 c.mm. O₂ per 0.0001 mg. Fe)
 0.0012 " " " " 71.5 " " (6.0 " " " 0.0001 " ")
 0.0008 " " " " 50.5 " " (6.3 " " " 0.0001 " ")
 0.0004 " " " " 25.5 " " (6.4 " " " 0.0001 " ")

which averages 6.2 c.mm. of O₂ per hour increase per 0.0001 mg. of Fe.

He has shown that the oxidation rate is nearly directly proportional to the amount of iron added, hence, if the residual oxidation is assumed to be due to the iron present as impurity, then there

should be present 0.000,056 mg. of Fe per 10 mg. of cysteine hydrochloride as determined by the following equation.

$$6.2 \text{ c.mm. O}_2 : 0.0001 \text{ mg. Fe} :: 3.47 \text{ c.mm. O}_2 : x \text{ mg. Fe}$$

$$x = 0.000,056 \text{ mg. Fe}$$

Then 0.1 gm. of cysteine hydrochloride should contain 0.00056 mg. of Fe, but he found that 0.1 gm. of his sample actually contained about 0.0001 mg. of Fe and certainly less than 0.0002 mg. of Fe. From this he concluded that the residual oxidation did not appear to be wholly due to the presence of iron, but since he failed to obtain similar results in his work with glutathione, which was reported in the second section of the same paper, no further consideration was given.

TABLE VI
Oxidation of Cysteine Plus Various Amounts of Iron

Amount of iron added	O ₂ uptake per hr	Increase in uptake per 0.0001 mg. Fe
<i>mg.</i>	<i>c mm.</i>	<i>c mm.</i>
0 0010	55 2	5 3
0 0008	45 5	5 2
0 0006	33 8	5 3
0 0004	23 6	5 1
0 0002	13 0	5 4
0 0001	7 2	5 0
No iron added	2 2	5 21 average

Figures represent uptake of oxygen per hour per 10 mg. sample of cysteine hydrochloride.

In order to determine the relation between the residual oxidation of the cysteine hydrochloride which was prepared by the author and which was shown to be exceedingly pure and the increased oxidation when iron was added, a series of experiments was carried out in which varying amounts of iron were added as FeCl₃. The results are given in Table VI and Fig. 1.

Fig. 1 shows the effect of adding increasing amounts of iron as ferric chloride on the rate of oxygen uptake of pure cysteine. The increase in rate is seen to be directly proportional to the quantity

of iron added. The initial rate of oxygen uptake for pure cysteine is 2.2 c.mm. of O_2 per hour per 10 mg. sample.

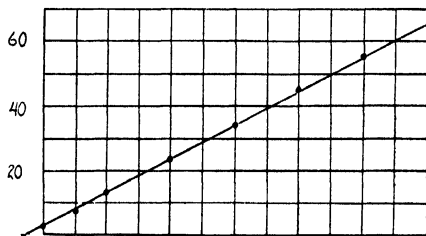


FIG. 1. The effect of concentration of iron on the oxidation of cysteine. Iron was added as $FeCl_3$; pH, 7.6; temperature, 25° . The distance AO represents about 0.45×10^{-4} mg. of iron, the amount necessary to account for the oxidation if it were due to traces of iron. The ordinate measures c.mm. of O_2 taken up per hour per 10 mg. of cysteine HCl; the abscissa, iron added in mg. $\times 10^{-4}$.

If (see Table II) this residual uptake is assumed to be due to a trace of iron remaining in the preparation, then, the rate of uptake calculated from values obtained when iron is added requires the presence of 0.000,044 mg. of iron according to the following relation.

$$5.2 \text{ c mm. } O_2 : 0.0001 \text{ mg. Fe} :: 2.2 \text{ c.mm } O_2 : x \text{ mg. Fe} \\ x = 0.000,044 \text{ mg. Fe}$$

On actual test, however, 10 mg. of the cysteine hydrochloride gave no test for iron and could contain at most but 0.000,001 mg. of Fe instead of the 0.000,044 mg. required by a consideration of the iron oxidation graph given in Fig. 1.

The average oxygen uptake per 10 mg. of cysteine hydrochloride obtained by Sakuma and Harrison is 1.22 and 3.47 c.mm. respectively, while the amounts of iron necessary to bring about this oxidation, if it is due to iron, are calculated to be 0.000,047 mg. and 0.000,054 mg. for the respective rates. Sakuma gives no quantitative results for iron in his preparation, while Harrison found that 10 mg. of his purest cysteine hydrochloride contained only 0.00001 mg. of iron.

The calculations show that the oxidation of neutralized cysteine

hydrochloride is much too rapid to be accounted for by the extremely minute and undetectable amounts of iron which might be, and possibly are, present in even the most pure preparation. The amount of iron which would be needed to account for the oxidation would be 44 times as much as could possibly be present. The results of Harrison and Sakuma indicate the same conclusion.

The author wishes to express his thanks to Professor A. P. Mathews, under whose direction this work was carried out.

SUMMARY AND CONCLUSION

Pure cysteine oxidizes spontaneously at a very slow rate, which is accelerated by the addition of very small amounts of iron roughly proportional to the amount of the metal added.

The rate of oxygen uptake of the pure cysteine is many times greater than could be attributed to traces of iron present.

It is concluded that pure cysteine free from iron is autoxidizable.

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STUDIES ON THE STARCH-IODINE REACTION

By JOHN FIELD

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The usefulness of the radiomicrometer in the study of reactions involving a marked color change was indicated in a previous paper (1). It was there shown that the starch-iodine reaction could be investigated in this way.

The present paper reports further observations on the reaction, which bear upon the problem of the chemical specificity of some starches and upon the nature of starch-iodide.

There exists a very extensive literature on the specificity of starches, much of which is noted in the exhaustive monograph of Reichert (2). The histological findings indicate that starch grains of any given plant have certain common characteristics, more or less distinctive of the family, genus, species, etc.

With regard to chemical specificity, matters are more unsettled. Differences in staining behavior, viscosity or plasticity, temperature range of gelatinization, etc., may be due to dissimilar physical states rather than to variations in chemical constitution. Elementary analysis has not evidenced differences between starches (2). Further, Ling and Nanji (3) have shown that the amylose : amylopectin ratio is practically constant in starches which do not contain hemicelluloses (4), while when hemicelluloses do occur, the hemicellulose content is much affected by the history of the preparation (5).

The starch-iodine reaction affords still another means of approach to the problem of the chemical specificity of starches. Since its discovery by Colin and de Claubry (6) in the laboratory of Gay-Lussac, this reaction has been the subject of numerous investigations. The blue color was regarded as specific from the time of Stromeier (7), until Claude Bernard showed that an abnormal glycogen may be formed in paralyzed muscles which will

give the same reaction (Reichert). The work of Barger and Field (8), and of Barger and Starling (9), has shown that a number of organic substances will react with iodine to produce blue adsorption compounds, the degree of dispersion being of importance in this connection.

While the formation of a blue iodide is not a unique property of starch, it might well be that one could distinguish between one starch and another by some peculiarity in the reaction with iodine. This was attempted by Dubosc (10), who placed iodine crystals on a watch-glass beside various kinds of starch grains, covered the whole with a bell jar, and allowed the system 24 hours to come to equilibrium. Dubosc reported that the several starch-iodides produced in this way showed some specificity in hue.

Diverse findings on the proportions of starch and of iodine in starch-iodide are to be found in the literature (11-16). The variation is such as to suggest that the reaction is not stoichiometric, and that some important factor or factors have not been uniformly controlled in the several investigations.

Starch solutions prepared after the methods of Alsberg and Rask (17) and of Alsberg, Griffing, and Field (18), which have been subjected to a minimum of chemical treatment, were employed in the following investigation. The concentrations of these were determined by the volumetric method of Munson and Walker (19). The glucose-starch factor recommended by the 1925 edition of Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists, *viz.* 0.9, was used in calculating the starch equivalent of the glucose determined.

A Leitz optical glass cuvette was used throughout as a titration vessel. In every case, 35 ml. aliquots of the starch solution (which was quite clear in the case of wheat and corn starches, but slightly opalescent when potato starch was used) were placed in this vessel. The solutions were thoroughly stirred after each addition of iodine.

All adjustments of diaphragms, focussing apparatus, etc., remained unchanged during the course of the experiments. Room temperature was practically constant at 16° during the runs on wheat and corn starches, and at 19° during the runs on potato.

The initial deflection of the radiomicrometer, with the titration vessel in place, was not significantly different from its deflection

in the absence of the titration vessel, with the intensity of radiant energy used. (The cooling cell, of course, was continuously present.)

DISCUSSION

The curves obtained by plotting values of the deflection of the radiomicrometer against the molar concentration of iodine at the several stages of titration are typified by Fig. 1. The shape of these curves is geometrically similar to that of the resonance curve when one starts from the resonance point. It is also similar to the right-hand branch of a probability curve. Such geometric

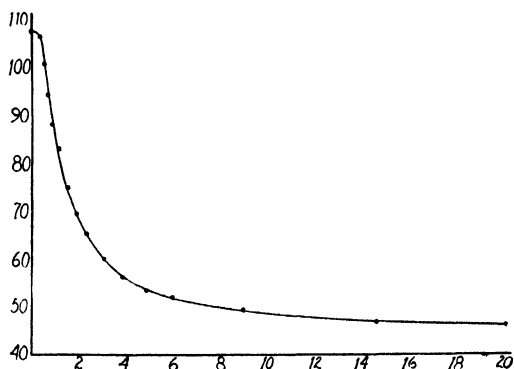


FIG. 1. Curve for corn-starch, Preparation 3. The deflection of the radiomicrometer (ordinate) is plotted against the molar concentration of iodine as $\text{mols} \times 10^{-6}$ (abscissa).

resemblance is interesting but does not warrant any definite physical conclusions.

The end-points of the several curves of deflection, plotted against mm of iodine added $\times 10^{-3}$, are not easily determined by inspection. If we add iodine to a volume of distilled water equal to the aliquot of starch solution used, the plotting of deflection against iodine should give a curve whose slope is the same as that of the tangent to the end-point of the starch curves. However, experimental error is most significant in this, the practically asymptotic portion of the curve, and end-points cannot be determined satisfactorily in this way ((20) p. 406, (21) p. 512).

While it is difficult to determine the end-point on the iodine axis, the end-point on the deflection axis can be located with considerable accuracy. This is analogous to the rheobase of Lapicque (21). If we assume that Beer's law holds for starch-iodide (22), the relative amounts of starch-iodide in the several preparations when these are saturated with iodine may be determined in the following way.

Let I denote the intensity of radiant energy incident upon the titration vessel, and t the intensity of the emergent radiant energy.

$$\text{Let } f = \frac{t}{I}.$$

Under the conditions of the experiments, we may write $I = t$ (within the limits of experimental error) before the titration begins. The values of t used in the calculations which follow are measured by the deflection of the radiomicrometer when the solution is saturated with iodine at the prevailing temperature, *i.e.* the value of deflection when the deflection-iodine curve has become asymptotic to a parallel to the x axis. These values were obtained graphically from the several curves.

By application of Lambert's law, f may be evaluated.

$$(1) \quad f^2 = 10^{-Acd}$$

where c is the concentration of the absorbent solution, d the distance through the solution along the path followed by the light, and A is a constant varying with the wave-length of the incident light (23).

Equation 1 may be rewritten in the form

$$(2) \quad f = 10^{-kc}$$

whence, by substitution,

$$\frac{T}{t} = 10^{kc}$$

or

$$c \propto \log_{10} \left(\frac{T}{t} \right)$$

TABLE I
Analysis of the Starch-Iodine Reactions

Series No.	T	t	$\frac{T}{t}$	$\text{Log } \frac{T}{t}$	Starch concentration mg. per cc.
Corn					
1	108.85	43.95	2.475	0.3936	8.7120
2	107.05	45.65	2.345	0.3701	2.7776
3	107.10	46.50	2.300	0.3617	1.9440
4	108.90	57.55	1.890	0.2765	0.8712
5	114.85	63.25	1.815	0.2589	0.5040
6	106.70	61.70	1.730	0.2380	0.3158
7	110.35	67.00	1.645	0.2161	0.3121
8	107.58	76.60	1.402	0.1467	0.1557
Wheat					
1	97.10	39.00	2.480	0.3945	5.0040
2	91.75	41.55	2.200	0.3424	1.6680
3	95.00	48.70	1.950	0.2900	0.6255
4	98.95	57.80	1.710	0.2330	0.2502
5	117.88	84.00	1.402	0.1467	0.1860
Potato					
1	69.80	26.00	2.680	0.4281	4.1472
2	69.40	27.10	2.560	0.4081	2.0736
3	68.35	28.50	2.400	0.3802	1.0368

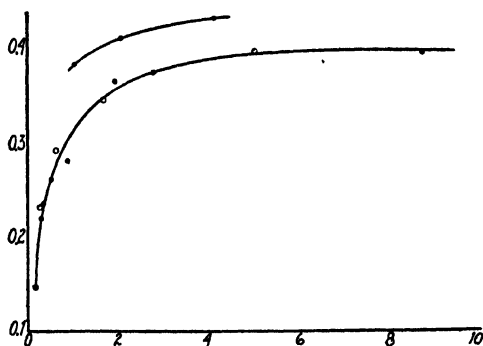


FIG. 2. $\text{Log}_{10} \frac{T}{t}$ (ordinate) plotted against the starch concentration measured in mg. per cc. (abscissa). The upper curve is for potato starch the lower for corn and wheat. On the lower curve the solid circles represent runs on corn-starch; the clear circles, runs on wheat.

Table I gives the values of T , t , $\frac{T}{t}$, and concentration of starch in mg. per cc. for the several experiments. In Fig. 2, $\log \frac{T}{t}$ is plotted against concentration for the several starches.

Table I and Figs. 1 and 2 show no significant quantitative difference in the taking up of iodine between the cold water-soluble portions of ground corn and wheat starches at comparable concentrations. The divergence in behavior shown by the preparations from potato starch may be due to its relative opalescence and to the change in room temperature.

The data also clearly indicate the importance of dilution in determining the amount of iodine taken up. For example, in the corn-starch preparations an increase in concentration from 15.57 mg. per cent to 871.2 mg. per cent, *i.e.* an increase of 5.600 per cent, involves an increase of only $\frac{0.3936}{0.1467} \times 100$ or 268 per cent in

the amount of starch-iodide present when the starch is saturated with iodine. This is strongly suggestive of adsorption, and tends to support the view of Freundlich, Küster, Padoa and Savare, Katayama, Lottermoser, Berzeller, and Bancroft that starch-iodide is an adsorption compound.

SUMMARY

Radiomicrometric titration has shown that clear solutions of the cold water-soluble fractions of ground corn and wheat starches, have, at a given concentration, approximately the same capacity for adsorbing iodine. This fact indicates a lack of specificity of these preparations in so far as this property is concerned.

The significance of dilution as a factor in determining the iodine-binding capacity of these preparations suggests that starch-iodide is an adsorption compound.

The writer's thanks are due to Doctor C. L. Alsberg, under whose direction the thesis of which this work is part was undertaken, and to Doctors L. G. M. B. Becking and L. S. Jacobsen for many helpful suggestions.

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STUDIES IN NUTRITIONAL ANEMIA
QUANTITATIVE VARIATIONS IN IRON, COPPER, AND
MANGANESE SUPPLEMENTS*

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(Received for publication, April 16, 1931)

The question of the optimum mineral supplement for hemoglobin regeneration in rats suffering from milk anemia has been attacked from several angles by different investigators. The first essential, an adequate supply of available iron, has most frequently been furnished by 0.5 mg. daily in the form of some soluble iron salt. Differences in response of anemic rats to this or similar quantities of iron have been attributed to possible contamination of the commercial salts with other metals (1), or to differences in availability of the various forms of iron used (2). With carefully purified iron salts Waddell and coworkers (3) failed to cure milk anemia in rats even when as high as 10 mg. of iron were fed daily. Beard and Myers (4), however, report a slow but steady increase in hemoglobin when pure iron salts furnished the only supplement to the milk ration.

The specificity of copper as a supplement to iron in hemoglobin regeneration has been demonstrated by Hart, Steenbock, *et al.* (5), Krauss (6), and Lewis *et al.* (7) while others maintain that although more effective than other minerals copper is not specific in this capacity. Our previous paper from this laboratory (2) also expressed this opinion. Drabkin and Waggoner (8) report copper as non-essential for hemoglobin regeneration, but are working with a synthetic ration which may introduce other factors not encountered in a simple milk anemia. Titus and Hughes (9), Goerner (10), and Beard and Myers (4) have attributed to manganese an

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effect similar to that of copper but somewhat less marked. The latter investigators have also found a variety of other minerals effective as supplements to iron in hemoglobin regeneration. Variations in experimental technique, strains of rats, milk supply, and other factors make it difficult to reconcile or explain the discrepancies in the results reported and the conclusions reached by these several investigators.

The present paper reports an extensive series of experiments undertaken in an attempt to throw further light on some of these questions. What is the nature of the response to iron alone at different levels of intake? Is manganese really effective as a supplement to iron in milk anemia? If so what is an optimum level of iron, copper, and manganese for the promptest and highest hemoglobin response? Are other metals present in the ash of plants significant in this capacity? Throughout this work emphasis has been placed on carefully standardized technique and a sufficiently large number of animals to overcome if possible the errors due to individual variations.

Procedure

Experimental Animals

Between 3000 and 4000 hemoglobin determinations on over 300 rats are reported in this paper. Many more used in preliminary tests are not included here. Severe secondary anemia was produced in young rats by the feeding of fresh milk from the time of weaning. To insure that none of the stock ration was consumed by the young while they were still with the mother, milk was the only food given to the family after the litter was 2 weeks old. A depletion period on milk alone averaging 5 to 6 weeks after weaning was usually sufficient to produce the desired degree of anemia. Fresh milk from a certified herd has been used throughout the experiment. Aluminum and block tin were the only metals which came in contact with the milk before it was fed. Rats have been kept in galvanized metal cages of which the raised screen bottoms and food cups were washed daily.

Hemoglobin Determinations

Hemoglobin determinations were made weekly on each animal until a normal level was reached after which biweekly determina-

tions were considered sufficient. The Newcomer method with a Bausch and Lomb hemoglobinometer with blue filter was employed throughout the experiment. A detailed discussion of the precautions observed is given in a subsequent article being published elsewhere (11).

Normal hemoglobin in our colony ranges between 15.3 and 16.6 gm. per 100 cc. of blood after the rats are 7 weeks of age. Complete regeneration is assumed when the hemoglobin level has reached 15 gm. or above.

Mineral Supplements

The iron, copper, and manganese salts used in these experiments were of the highest grade obtainable (Baker's analyzed) and were further subjected to most careful purification in this laboratory. Iron obtained as ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) was dissolved in double distilled water and subjected to H_2S precipitation, after the acidity of the solution had been adjusted to 0.3 N. The salt was crystallized from the filtrate, redissolved, and again subjected to H_2S precipitation. Three subsequent recrystallizations from double distilled water yielded the salt which was finally used for the feeding tests. This procedure proved more satisfactory and yielded a product of greater purity than that prepared from iron wire. Manganous sulfate ($\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$) was treated in the same manner as the iron salt. Copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) was recrystallized five times from double distilled water. All water used in these manipulations and in solutions for feeding was distilled the second time from a complete glass apparatus to avoid all mineral contamination. Table I shows the various amounts and combinations of iron, copper, and manganese fed as daily doses 6 days a week.

The solutions of salts were prepared in such dilutions that 0.2, 0.5, 1, or 2 cc. daily would supply the desired amounts of the minerals. Such volumes were easily measured and did not greatly reduce the concentration of the milk fed. The supplements were added to the morning feeding which was small in amount and was consequently completely consumed within a few hours. Later in the day additional milk was given *ad libitum*.

In one short series of tests salts of aluminum, tin, antimony, and zinc were fed as supplements to 0.5 mg. of iron. The unquestion-

ably negative results obtained in every case prompted us to concentrate attention on the iron, copper, and manganese only. These findings also reassured us that the metals with which the milk or the rats came in contact were not serious sources of error in the present investigation.

TABLE I

Quantities and Combinations of Iron, Copper, and Manganese Fed as Supplements to a Milk Ration

Iron (ferrous sulfate)	Copper (copper sulfate)					Manganese (manganous sulfate)			
<i>mg. per day</i>	<i>mg. per day</i>					<i>mg. per day</i>			
0.5	0.1	0.05	0.025	0.01					
0.25	0.1	0.05	0.025	0.01					
0.1	0.1	0.05	0.025	0.01					
0.5						0.1	0.05		
0.25						0.1			
0.1						0.1			
0.5	0.1					0.1	0.05	0.025	0.01
0.5		0.05				0.1	0.05	0.025	0.01
0.5			0.025			0.1	0.05	0.025	0.01
0.5				0.01		0.1	0.05	0.025	0.01
0.25	0.1					0.1	0.05	0.025	0.01
0.25		0.05				0.1	0.05	0.025	0.01
0.25			0.025			0.1	0.05	0.025	0.01
0.25				0.01		0.1	0.05	0.025	0.01
0.1	0.1					0.1	0.05	0.025	0.01
0.1		0.05				0.1	0.05	0.025	0.01
0.1			0.025			0.1	0.05	0.025	0.01
0.1				0.01		0.1	0.05	0.025	0.01
0.5	1	2	4	6	8				
0.5						1	2	4	

Initial Hemoglobin

A uniformly low hemoglobin at the time when mineral additions were to be made was found to be of prime importance. Only under such conditions could it be assumed that the physiological stores of the essential elements had been equally depleted. It frequently happened that one or two rats in a litter failed to develop as severe anemia in a given time as had the litter mates. As it was believed that a uniform age for supplementing the milk ration of litter mates was desirable in some of our earlier work, these less anemic rats had received the mineral additions at the

same time as the majority which were more anemic. The prompter recovery of those which started at a higher level made us realize that a low initial hemoglobin was of far more importance for consistent results than the exact age of the animal. Without this precaution the hemoglobin responses to the same or to graded quantities of mineral supplements were neither constant nor consistent. At best there is a natural variation in animals which must be taken into account. Table II shows the hemoglobin response over a period of 10 weeks of two groups of rats, receiving exactly the same mineral supplement (0.5 mg. of Fe), the only difference between the groups being that the initial hemoglobin values in the first ranged between 4.5 and 6 gm. per 100 cc. of

TABLE II

Weekly Hemoglobin Averages Showing Difference in Response Due to Initial Hemoglobin Level

The Hb values are given in gm. per 100 cc. of blood for rats fed milk plus 0.5 mg. of Fe daily.

Initial Hb between	Initial Hb	Week of experimental period									
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
4.5-6 0	5 6	7.4	8.9	10.3	11.3	11.7	13.1	13.1	13.6	14.1	13.8
3.0-4.5	3.3	4 3	5.8	6.1	7.1	8.2	8.3	8 8	9.4	10.9	11.2
Difference due to initial Hb.....	2.3	2 9	3.1	4.2	4.2	3.5	4.8	4.3	4.2	3.2	2 6

blood while in the second they ranged from 3 to 4.5 gm. This initial difference is reflected in the later response in hemoglobin level. All animals which had shown an initial hemoglobin of more than 4.5 gm. were subsequently discarded from our data as soon as the importance of this precaution was observed. The difference in speed of hemoglobin regeneration due to such initial variation may easily have been responsible for the inconsistent results and different conclusions reported by other workers. In an early paper from the Wisconsin (12) laboratories 4.23 ± 0.15 gm. of hemoglobin per 100 cc. of blood were recommended as a suitable degree of anemia for a starting point, but in the next series of papers initial hemoglobin values ranging from 3.0 to 10.0 were apparently considered satisfactory. In more recent work,

however, a lower and narrower range has again been employed. Exact figures are not always given in the published reports but the approximate range (calculated from graphs or figures) of the initial hemoglobin values used by workers of several other laboratories may be significant.

Beard and Myers (4).....	4.0-6.0 gm. per 100 cc.
Titus and Hughes (9)	2.3-9.0 " " 100 "
Drabkin and Waggoner (8)	5.5-8.0 " " 100 "
Krauss (6).....	2.0-5.0 " " 100 "
Lewis <i>et al.</i> (7).....	3.0-6.75 " " 100 "
McGhee (13).....	75 per cent (standard not given)

We wish to emphasize the necessity of uniformly low initial hemoglobin values in experimental animals when any factor purported to have a stimulating effect on hemoglobin synthesis is being investigated.

DISCUSSION

Space does not permit the publication in detail of the large volume of data accumulated during these experiments but composite tables and charts afford a means of summarizing the results and of arriving at fair and unprejudiced interpretation thereof.

Response Due to Iron Alone

The feeding of a pure iron salt as the only supplement to milk has in the present series as well as in our previous experience resulted in a very slow but unmistakable increase in hemoglobin concentration. In the present instance when the iron salt was of such purity as to preclude possibility of significant copper contamination it seems most logical to attribute the slow response observed to the traces of copper available from the milk supply or stored in the animal body. As the former item may vary in different localities it is at least a plausible explanation of the variations observed in several laboratories concerning the ability of the rat to synthesize hemoglobin on a ration of milk and a pure iron salt. The average difference in the hemoglobin response to three levels of iron (0.5, 0.25, and 0.1 mg. daily) is shown in Chart I and Table III.

Response Due to Iron and Copper

The rapid synthesis of hemoglobin when even the lowest level of copper (0.01 mg. daily) was fed in addition to 0.5 mg. of iron confirmed our previous results as well as those of several other

TABLE III

Weekly Hemoglobin Averages on Different Levels of Iron and Copper Supplements

The Hb values are given in gm. per 100 cc. of blood.

Supplement		No. of rats	Initial Hb	Average weekly Hb for									
Fe	Cu			1st wk.	2nd wk.	3rd wk.	4th wk.	5th wk.	6th wk.	7th wk.	8th wk.	9th wk.	10th wk.
mg.	mg.												
0.5		6	3.6	5.3	8.0	8.1	9.1	8.9	9.2	9.9	10.4	12.0	11.6
0.25		5	4.2	4.0	4.4	5.1	5.1	5.8	6.4	6.7	7.3	8.6	8.7
0.1		6	3.8	3.7	4.2	4.7	4.6	4.9	5.5	6.0	6.0	6.2	7.0
0.5	0.1	8	3.8	8.9	11.8	14.1	14.5	15.9	15.6	15.7	15.7	15.7	15.5
0.5	0.05	8	3.8	9.1	12.8	13.9	14.1	14.9	14.7	14.7	14.5	14.9	14.9
0.5	0.025	4	3.8	8.3	13.8	13.2	13.5	15.7	16.4	15.9	16.3	15.9	15.5
0.5	0.01	6	3.8	7.3	10.6	12.2	13.3	14.8	14.9	15.0	15.3	16.0	15.6
0.25	0.1	7	3.9	7.5	10.8	13.2	14.2	15.2	16.0	16.0	15.9	15.9	16.1
0.25	0.05	4	3.8	6.3	9.0	10.9	13.2	14.6	14.3	15.7	15.6	15.1	15.4
0.25	0.025	5	3.7	8.7	11.6	13.4	14.2	15.9	16.0	15.9	15.9	15.9	16.2
0.25	0.01	4	3.3	5.3	7.2	8.7	10.1	11.4	13.3	13.4	14.3	14.3	12.6
0.1	0.1	4	3.6	6.5	8.5	10.2	11.8	13.3	14.4	15.2	15.8	15.9	15.9
0.1	0.05	4	3.8	6.4	8.4	9.9	11.0	11.3	13.0	13.5	14.3	14.4	15.1
0.1	0.025	4	3.5	5.3	7.1	8.5	9.2	9.9	10.9	12.0	12.6	12.7	13.3
0.1	0.01	5	3.8	4.8	5.7	6.8	7.3	8.3	9.2	10.3	11.2	11.3	11.7
0.5	1.0	7	3.8	9.6	13.6	14.4	15.3	17.0	16.6	16.4	16.5	17.0	16.5
0.5	2.0	4	3.7	10.1	14.9	14.5	15.1	14.7	15.0	15.2	15.1	14.9	14.7
0.5	4.0	7	3.6	8.6	12.8	12.6	15.2	14.8	14.9	14.9	15.1	14.6	14.4
0.5	6.0	5	3.4	9.1	12.4	14.8	14.7	16.2	16.1	14.9	14.9	14.2	13.7
0.5	8.0	5	3.8	8.5	14.9	15.2	16.3	16.3	16.1	15.2	14.8	15.4	16.0

investigators that copper is exceedingly potent as a supplement to iron. Table III gives the averages for the first series of iron and copper tests. The full significance of Chart I which summarizes these results along with other data subsequently available will be

discussed later. Numerous tests were made with 0.1, 0.05, 0.025, and 0.01 mg. of copper daily. Larger quantities of copper (1, 2, 4, 6, and 8 mg. daily) were also fed to determine whether any additional increment of response or possible toxic effects might be noted. Rats refuse to drink milk containing high concentrations of copper. Attempts at dilution with larger volumes of milk often resulted in incomplete consumption of the total mineral dosage. Thus results from this latter group must be considered approximate but at least no toxic effect was noted. From these data it may be concluded that the optimum level of copper for the rat is between 0.1 and 1.0 mg. daily. No increase in rate or height of hemoglobin regeneration was observed with amounts greater than 1.0 mg. but rather was there a suggestion of less rapid response with the higher levels of copper. With daily doses of less than 0.1 mg. there was slight but distinct retardation in hemoglobin synthesis which was more evident when the daily iron allowance was low (0.25 or 0.1 mg. daily).

Response Due to Iron, Copper, and Manganese

The various doses of manganese noted in Table I were fed as supplements to each of the quantities of iron and to each of the combinations of iron and copper. It was conceivable that some combination of these three elements might result in optimum hemoglobin synthesis. 211 rats in all were fed on the four doses of manganese (0.1, 0.05, 0.025, and 0.01 mg. daily). The apparently inconsistent and variable results obtained led us to analyze these data from every possible angle. Individual variation in animals is so great as to necessitate very appreciable differences before conclusions may be drawn. The apparent slight beneficial effect of manganese noted in some of our earlier work and in those rats receiving higher levels of manganese (1, 2, and 4 mg. daily) was observed in a limited number of cases in all of which a uniformly low initial hemoglobin did not obtain. Further experiments are being conducted on this phase of the problem but difficulty has repeatedly been encountered in inducing rats to take the higher doses of manganese. As in the case of higher copper levels results are unreliable when incomplete mineral supplement and less than an adequate quantity of milk are consumed.

The responses of groups of rats receiving different mineral sup-

plements may conveniently be compared by subtracting the average figures week by week for gm. of hemoglobin per 100 cc. of blood. These differences may be interpreted as due to the difference in mineral supplement supplied. If such a comparison is made between differences due to copper and those due to manganese at exactly the same level of intake the differences due to manganese are much less than those due to copper. But it might

TABLE IV

Weekly Hemoglobin Averages Showing Response to Different Levels of Manganese with Constant Iron and Copper Supplements

The Hb values are given in gm. per 100 cc. of blood.

Supplement to milk ration			No. of animals	Initial Hb	Average weekly Hb for									
Fe	Cu	Mn			1st wk.	2nd wk.	3rd wk.	4th wk.	5th wk.	6th wk.	7th wk.	8th wk.	9th wk.	10th wk.
mg.	mg.	mg.												
0.5	0.01	0.1	4	3.7	8.3	11.7	13.7	15.6	15.6	15.6	15.2	15.4	15.6	16.7
0.5	0.01		6	3.8	7.3	10.6	12.2	13.3	14.8	14.9	15.0	15.3	16.0	15.6
Difference.				-0.1	1.0	1.1	1.5	2.3	0.8	0.7	0.2	0.1	-0.4	1.1
0.5	0.01	0.05	4	3.2	8.5	11.8	14.0	14.6	14.8	15.3	15.7	15.6	16.0	16.5
0.5	0.01		6	3.8	7.3	10.6	12.2	13.3	14.8	14.9	15.0	15.3	16.0	15.6
Difference.				-0.6	1.2	1.2	1.8	1.3	0	0.4	0.7	0.3	0	0.9
0.5	0.01	0.025	5	3.4	7.7	11.4	13.1	14.5	15.0	15.4	14.3	15.3	15.6	15.8
0.5	0.01		6	3.8	7.3	10.6	12.2	13.3	14.8	14.9	15.0	15.3	16.0	15.6
Difference.				-0.4	0.4	0.8	0.9	1.2	0.2	0.5	-0.7	0	-0.4	0.2
0.5	0.01	0.01	4	3.3	7.9	12.5	14.5	14.9	15.8	16.5	16.1	15.6	15.9	16.1
0.5	0.01		6	3.8	7.3	10.6	12.2	13.3	14.8	14.9	15.0	15.3	16.0	15.6
Difference.				-0.5	0.6	1.9	2.3	1.6	1.0	1.6	1.1	0.3	-0.1	0.5

be argued that these smaller differences were still significant if it were not for the fact that a volume of additional data does not substantiate even this slight beneficial effect. A similar calculation showing the effect of four levels of manganese fed as supplements to 0.5 mg. of iron and 0.01 mg. of copper is given in Table IV. This low level of copper would presumably give manganese a chance to show its effect if there were any. The differences due

to manganese in this instance seem to be negligible as they fall largely within the range of experimental error. More convincing, however, than either of these is the evidence presented in Table V. The differences listed here are those due to 0.1 mg. of manganese daily fed in addition to the iron and copper doses listed in the left-hand column. Since there is no consistent or significant variation correlated with the graded manganese dosages nor any summation effect due to manganese plus copper it seemed logical to take the algebraic total of these differences to detect if possible any general trend. The average difference due to 0.1 mg. of manganese obtained by this calculation resolves itself into distinct insignificance. Three similar tables, not published here, demonstrate that the lesser doses of manganese (0.05, 0.025, and 0.01 mg. daily) are equally ineffective in stimulating hemoglobin regeneration in milk anemia in rats. It may therefore be concluded from these data that manganese, in doses of 0.1, 0.05, 0.025, and 0.01 mg. daily, has a negligible or at least insignificant effect in the synthesis of hemoglobin in the rat, either when used alone with iron or in addition to iron and copper.

It must be remarked, however, that manganese appears to have some stimulating effect on growth and food intake; and it yet remains to be demonstrated what physiological effects the higher levels of manganese may have if the rats can be forced to take these amounts.

Assuming then that the effect of these levels of manganese on hemoglobin regeneration is negative, we are justified in using all of the data accumulated in the groups fed on iron, copper, and manganese for making a more extensive study of the response of rats to graded quantities of iron and copper disregarding the manganese additions. Chart I represents the averages of the larger number of rats thus available on each of the levels of iron and copper indicated. Over 900 hemoglobin determinations are represented in each of the three groups of curves. A careful study of this chart reveals several interesting facts. With 0.5 mg. of iron even the lowest of the four levels of Cu is almost as effective as the highest level in its supplementary action. The contrast is striking, however, between the response to 0.01 mg. of copper and that to 0.5 mg. of iron. With half the quantity of iron (0.25 mg. daily) the higher doses of copper succeed in stimulating a synthesis of

TABLE V
Differences in Hemoglobin Levels Due to 0.1 Mg. of Manganese Fed in Addition to Various Quantities of Iron and Copper Supplements

The Hb values are given in gm. per 100 cc. of blood.

Supplements		No. of rats	Initial difference	Week of experimental period									
Fe	Cu			1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
mg.	mg.												
0.5		11	0.4	1.2	0.1	1.5	1.1	1.1	1.0	1.1	1.4	1.0	0.9
0.5	0.1	12	-0.2	-0.2	-1.0	-3.1	1.2	1.6	1.6	1.2	1.0	0.9	1.2
0.5	0.05	12	-0.1	0	-0.1	0.1	1.7	0.2	1.0	1.6	1.4	1.0	1.5
0.5	0.025	8	-0.1	-0.5	-1.9	0.8	1.3	-0.1	0.6	1.1	0.7	0.9	1.7
0.5	0.01	10	-0.1	1.0	1.1	1.5	2.3	0.8	0.7	0.2	0.1	-0.4	1.1
0.25	0.1	11	-0.1	-0.2	-0.1	-1.0	-0.8	-0.7	-0.5	-0.4	-0.1	0.7	-0.1
0.25	0.05	8	-0.3	0.8	1.6	2.1	0.6	0.4	1.3	0.1	-0.3	0.9	0.1
0.25	0.025	9	0.2	-3.9	-3.9	-1.6	-2.3	-2.9	-2.4	-1.6	-0.9	-0.6	-0.2
0.25	0.01	8	0.3	-0.7	-0.1	0.5	0.9	1.6	0.3	0.8	0.3	1.0	2.7
0.1		9	-0.2	0.6	0.3	0.4	0.8	0.3	-0.3	-0.6	-0.6	0.4	-0.7
0.1	0.1	9	0.3	-0.9	-1.3	-2.1	-2.1	-3.0	-2.4	-2.6	-0.9	-0.8	-2.0
0.1	0.05	9	-0.2	-1.0	-1.8	-2.3	-1.8	-0.5	-1.3	-1.5	-1.4	-1.0	-0.3
0.1	0.025	10	0.3	0.6	1.4	2.6	3.0	3.8	2.9	2.2	1.4	2.2	2.2
0.1	0.01	10	-0.3	-0.8	-0.7	-0.6	-0.1	-0.7	-1.2	-1.6	-3.0	-3.0	-3.7
Algebraic total.....			-0.1	-3.7	-6.4	-1.2	5.8	1.9	1.3	0	-0.9	3.2	4.4
Average difference.....			0	-0.3	-0.5	-0.1	0.4	0.1	0.1	0	-0.1	0.2	0.3

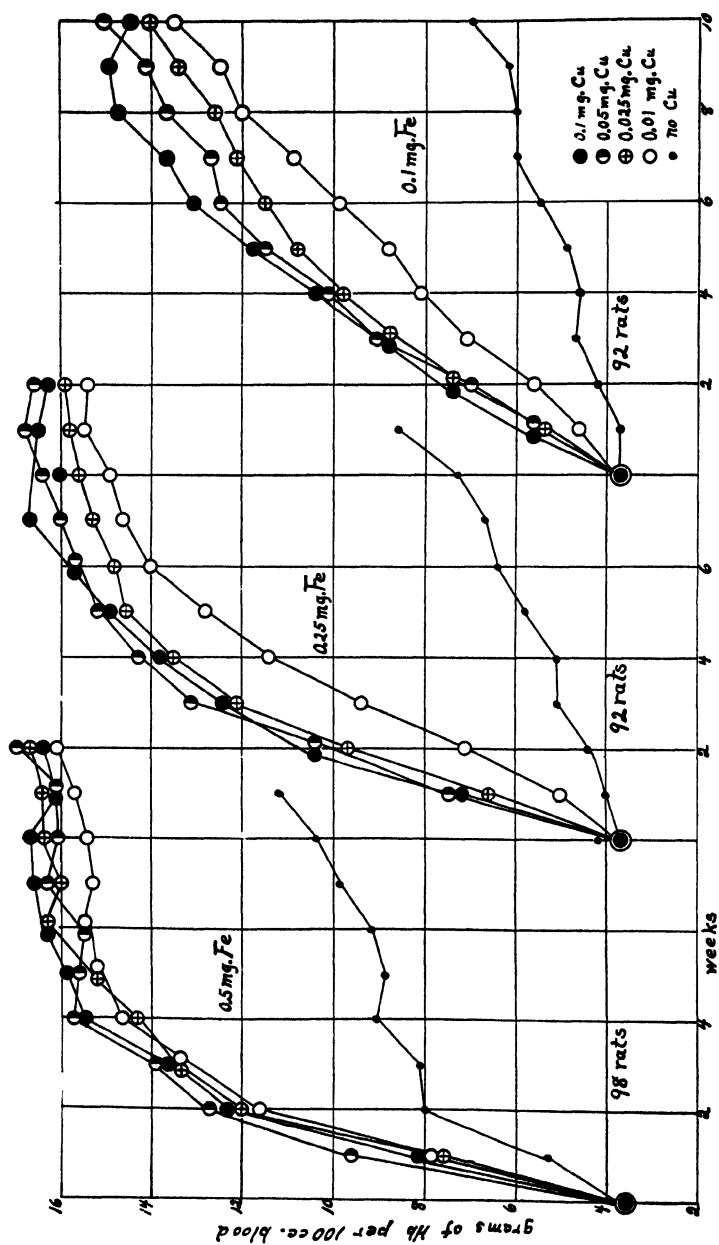


CHART I. Average hemoglobin response to different levels of intake of iron and copper supplements to milk

hemoglobin to the normal level in 8 weeks while on the smaller doses the normal level is not reached in 10 weeks. The difference between 0.01 mg. of copper and none, however, is more pronounced than with the 0.5 mg. of iron.

With 0.1 mg. of iron the differences in supplementary action of the various doses of copper are even more pronounced and the slope of all the curves has changed approaching a straight line. The natural tendency for rapid recovery followed by a plateau at the normal hemoglobin level has been partially inhibited. This would indicate that the iron as well as the copper has become a limiting factor at this level. Furthermore, none of these averages reaches the normal during the whole experimental period of 10 weeks. It may therefore be concluded that 0.1 mg. of iron daily is decidedly inadequate for the growing rat although it may serve as a convenient device for demonstrating the slight differences in response due to a so called catalytic supplement such as copper.

Comparing these three groups of curves it would appear that 0.25 mg. of Fe daily is adequate for the rat if sufficient copper is present in the ration. At this level also the differences are easily noted between the various doses of copper and none. It is therefore suggested that 0.25 mg. of iron daily is a convenient level to use when testing the supplementary action of other minerals, food, etc.

SUMMARY

1. There is slow but definite hemoglobin response to pure iron salts. The response is directly proportional to the amount of iron fed within the limits tested in this experiment.

2. The supplementary action of copper is confirmed. The optimum daily dose for a rat appears to be between 0.1 and 1.0 mg. daily.

3. Manganese fed in 0.1, 0.05, 0.025, and 0.01 mg. doses daily has a negligible supplementary action to iron in hemoglobin synthesis, but may have a slight stimulating effect on growth and food intake. Higher doses of manganese are being investigated further.

4. The optimum daily iron requirement for a rat is around 0.25 mg. if sufficient copper supplement (0.1 mg. daily) is provided. The 0.5 mg. of Fe daily used by many investigators affords a

margin of excess which, however, may not be desirable when differences in the supplementary action of graded doses of other factors are being demonstrated.

5. When recovery from a nutritional anemia is the experimental procedure a uniformly low initial hemoglobin in experimental animals is essential for consistent results.

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THE EFFECT OF LACTOSE AND THE ACID-BASE VALUE OF THE DIET ON THE HYDROGEN ION CONCENTRATION OF THE INTESTINAL CONTENTS OF THE RAT AND THEIR POSSIBLE INFLUENCE ON CALCIUM ABSORPTION*

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The effect of lactose on intestinal reaction assumed importance when the investigations of Kendall (1), Torrey (2), Retger and Cheplin (3), Cruickshank (4), and others showed that the ingestion of lactose resulted in a predominantly aciduric flora in the intestines. The usual explanation advanced has been that the large amount of carbohydrate in the intestinal contents enabled acid-forming organisms to thrive and to produce a degree of acidity which they alone could tolerate.

Subsequent work indicated that the simultaneous feeding of lactose increased the absorption of calcium from ingested calcium compounds (5, 6), raised the blood calcium (7), and relieved the symptoms of parathyroid tetany (8, 9). The question has arisen as to whether or not these phenomena are the results of the increased acidity of the intestinal contents.

Attention has already been called (6) to the paucity of evidence in support of the assumption that increased acidity, if it exists, increases calcium absorption. At the present time the actual evidence that there is even a significant increase in acidity is meager. Thus Retger and Cheplin (3) were unable to demonstrate in rats a consistent change in the reaction of the mixed feces. One of us (10) obtained similar results with human material but was able to show that, while the normal range was not

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exceeded, feces from subjects on a heavy lactose diet had a tendency to increase slightly in acidity in passing through the colon, whereas otherwise such material became somewhat more alkaline.

Dragstedt and Peacock (8) found that dogs on a diet of boiled rice, beef heart, and lactose did not produce acid stools although on a ration of bread, milk, and lactose the reverse was true. It should be noted however that in the former case the stools were not liquid while in the latter case they were. One of us (10) has shown that when a diarrheal condition exists acid stools result, which finding was confirmed by Schaudt (11).

McClendon, Myers, Culligan, and Gydesen (12) report a decrease of doubtful magnitude in pH of the ileal contents of cats and rabbits but inadequate controls make the results only suggestive. Cannon and McNease (13) appear to have clear cut evidence that the normal reaction of 7.0 to 7.1 of the cecum and colon of rats on a raw meat diet was reduced to 4.4 to 4.8 for the cecum and 6.0 for the colon when lactose was fed with meat. Beach (14) found a reduction from 6.0 to 7.4, to 4.4 to 5.6 of the cecal contents of chickens. In some patients observed by Paulson (15) the contents of the lower ileum were somewhat less alkaline under the influence of lactose feeding.

Whatever significance these results may have is greatly reduced when it is recalled that, as Redman, Willmot, and Wokes (16) and others have pointed out, the active absorption of minerals probably takes place in the small intestine and only to a slight extent, if at all, in the large intestine.

Results of studies of the acid-base value of food in relation to intestinal reaction are not at all consistent. McClendon and his coworkers (12) concluded with respect to the ileal content of rabbits that "The mineral content of the food does not seem to be a factor in determining the pH." One of us (10) found with human subjects that magnesium hydroxide caused a rise in fecal pH after the cathartic effect had passed. Schaudt (11) was unable to demonstrate a change in human fecal reaction after the administration of 20.0 gm. of NaHCO_3 by mouth or 40.0 cc. of N solution intravenously. NaH_2PO_4 by mouth gave an acid stool but the element of catharsis may have complicated this result. Helzer (17) found that ingested acid or alkali caused a corresponding excretion of hydrogen or hydroxyl ion into the gut apparently as a part of the adjustment of the acid-base balance in the body.

Shohl and Bing (18) secured acid feces from rats on a diet made acid with acid phosphate and, as did Zucker and Matzner (19) and Jephcott and Bacharach (20), from a basic diet plus cod liver oil. Otherwise alkaline feces followed basic diets.

In the present work the attempt has been made to ascertain the effect of low and high levels of lactose feeding on the reaction of intestinal contents from the lower duodenum through the colon

TABLE I
Composition of Feeds

	Diet A		Diet B		Diet C	
Protein, per cent.	19.9		47.5		46.6	
Fat (exclusive of cod liver oil), per cent.	4.4		8.4		7.8	
	(a)	(b)	(a)	(b)	(a)	(b)
Ca.	0.41	205	1.08	540	5.26	2630
Mg.	0.30	250	0.16	133	0.12	99
Na.	0.57	248	0.53	231	0.71	309
K.	0.55	141	0.42	108	0.60	154
Total base (+).		844		1012		3192
P*.	0.47	273	0.97	563	2.54	1474
S.	0.77	481	0.48	300	0.36	225
Cl.	0.60	169	0.65	183	0.44	124
Total acid (-).		923		1046		1823
Excess.		-79		-34		+1369

(a) = gm. per 100 gm. of feed; (b) = cc. of 0.1 N solution per 100 gm. of feed.

* Calculated as having a valence of 1.8 (21) to make the results comparable to published values for diets used in metabolism studies of rickets (22). A valence of 3 being used, the values for "Excess" become -260, -398, and +388 cc. of 0.1 N solution respectively.

with a vegetable diet and with one high in animal protein, to determine how much, if at all, the acidity is increased and whether such increase occurs in the region where absorption presumably takes place. A study was also made of the effect of increasing the base value of the high protein diet. Enough animals have been used so that the significance of variations in results could be determined statistically.

EXPERIMENTAL

Two types of diets were used. Diet A was a regular stock diet consisting of ground grain and alfalfa to which was added cod liver oil. The other type was prepared from ground dog biscuit and cooked meat dried and ground. Two lots of the latter were prepared, Diet B having an excess of acid and Diet C having an excess of base. This was probably in the form of ground bone in the meat as the calcium and phosphorus in Diet C were much higher than in Diet B. The analyses are shown in Table I.

Groups of about twenty-five mixed white and piebald rats each received respectively these three basal rations alone, basal rations Diet A and Diet B plus 5 per cent lactose, and the three basal rations plus 25 per cent lactose. Normal stools were produced by all of the diets which were fed for at least 2 weeks. The animals were killed by a sharp blow on the head at least $1\frac{1}{2}$ hours after feeding. The gastrointestinal tract was removed and tied off into the following sections; stomach, duodenum, three equal lengths of the small intestine, the cecum, and the colon. The contents were expressed into small tubes with the addition of the minimum amount of water to give, after centrifuging, a drop of material sufficiently fluid to be drawn up into a Cullen quinhydrone electrode (23). The readings were made at room temperatures against a saturated calomel cell with a saturated KCl bridge. Corrections were made for variations in temperature in calculating the pH (24). The electrodes were checked frequently against 0.1 N HCl and no trouble was experienced in making readings except at some of the higher values above pH 8.0. The collection of suitable samples from the stomach and duodenum proved so difficult that these results were discarded. As a rule the material in the duodenum was so small in quantity and of such consistency—frequently only a shred of heavy mucus—that an accurate reading was impossible, while the stomach was either empty or filled with a mass of dry undigested food which required the addition of so much water that the results could not be trusted. To test the accuracy of the application to intestinal contents of the method finally used, several samples were run with both the quinhydrone and the hydrogen electrode. Table II gives the results.

Experiments on five to nine animals were run in half a day,

about 45 minutes elapsing between the killing of the animal and the completion of its examination. However as they were all fed

TABLE II
Comparison of Quinhydrone and Hydrogen Electrode

Section of gastrointes- tinal tract*	Diet C			Section of gastrointes- tinal tract*	Diet C + lactose		
	Quinhy- drone electrode	Hydrogen electrode	Difference		Quinhy- drone electrode	Hydrogen electrode	Difference
S	2.36	2.27	0.09	S	5.04	5.01	0.03
II	7.01	6.81	0.20	I	6.61	6.54	0.07
III	7.04	6.97	0.07	II	6.87	6.69	0.18
Ce	7.06	7.22	0.16	III	7.02	6.97	0.05
Co	7.17	7.18	0.01	Ce	6.15	6.23	0.08
				Co	7.31	7.33	0.02
S	5.10	5.11	0.01	S	5.45	5.53	0.08
II	6.58	6.58	0.00	I	6.38	6.34	0.04
III	7.22	7.25	0.03	II	6.77	6.70	0.07
Ce	7.17	7.22	0.05	III	7.02	7.02	0.00
Co	7.19	7.22	0.03	Ce	6.33	6.32	0.01
				Co	6.92	7.04	0.12
S	4.86	4.71	0.15	S	4.79	4.83	0.04
I	6.17	6.23	0.06	I	6.19	6.18	0.01
II	6.75	6.70	0.05	II	6.27	6.29	0.02
III	7.42	7.45	0.03	III	6.07	6.07	0.00
Ce	7.14	7.23	0.09	Ce	7.00	6.82	0.18
Co	6.96	7.04	0.08	Co	7.23	7.19	0.04
S	3.82	3.80	0.02	S	5.19	5.18	0.01
I	6.50	6.32	0.18	I	6.62	6.62	0.00
II	6.88	6.75	0.13	II	6.83	6.80	0.03
III	7.50	7.56	0.06	III	7.28	7.23	0.05
Ce	7.03	7.17	0.14	Ce	6.27	6.33	0.06
Co	7.36	7.26	0.10	Co	6.67	6.74	0.07

* The gastrointestinal tract was divided into the following sections: S, stomach; I, II, and III, equal lengths of the small intestine; Ce, cecum; and Co, colon.

at the same time the results on the last rat were secured about 4 hours after feeding as compared with an hour and a half for the first one examined. Hence the figures include values secured over

the whole period of most active digestion. Plotting the first and last figures revealed no characteristic differences due to the progress of digestion. This finding is in agreement with that of Redman, Willmot, and Wokes (16).

DISCUSSION

The data in Fig. 1 are summarized in Tables III and IV. This shows the means, differences of the means, and probable errors

TABLE III

Effect of Lactose on Intestinal Reactions with High and Low Protein Diets

Section of gastroin- testinal tract	Lac- tose added	Low protein diet	Means	Mean devia- tion	Prob- able error	High protein diet	Means	Mean devia- tion	Prob- able error
	per cent								
I*	5	A	6.75-6.64	-0.11	±0.05	B	6.42-6.46	+0.04	±0.06
	25	"	6.75-6.53	-0.22	±0.04	"	6.42-6.44	+0.02	±0.04
	25					C	6.38-6.55	+0.17	±0.03
II*	5	"	7.70-7.51	-0.19	±0.07	B	6.85-6.77	-0.08	±0.04
	25	"	7.70-6.98	-0.72	±0.06	"	6.85-6.66	-0.19	±0.13
	25					C	6.80-6.91	+0.11	±0.05
III*	5	"	8.19-7.97	-0.22	±0.05	B	7.34-7.18	-0.16	±0.06
	25	"	8.19-7.39	-0.80	±0.05	"	7.34-6.86	-0.48	±0.06
	25					C	7.26-6.99	-0.27	±0.05
Cecum	5	"	7.04-6.81	-0.23	±0.04	B	7.28-7.28	0.00	
	25	"	7.04-5.99	-1.05	±0.06	"	7.28-6.94	-0.34	±0.07
	25					C	7.06-6.59	-0.47	±0.06
Colon	5	"	7.18-6.88	-0.30	±0.04	B	7.22-7.29	+0.07	±0.05
	25	"	7.18-6.61	-0.57	±0.06	"	7.22-7.09	-0.13	±0.05
	25					C	7.11-7.00	-0.11	±0.05

* The small intestine was divided into three equal sections.

of these differences, calculated by the formula $PE_m = 0.6745 \sqrt{\frac{\sum d^2}{n^2}}$

where d and n represent respectively the difference of the means and the number of samples examined.

Normal Reaction—There are in the literature many values for the intestinal reaction of rats on various diets, but the variety of these diets, the methods of determining the hydrogen ion concentration, and other considerations make accurate comparisons difficult.

Our results with the vegetable diet agree with those of McRobert (25) and of Schoubye (26) who used similar diets in that they show a maximum pH which is in the alkaline range in the region of the ileum. Our results for the upper small intestine and the colon fall within the range observed for these sections by Tisdall and Price (27) but for the lower small intestine and cecum we observed much higher values. McRobert suggests that the use of an anesthetic for killing the rats caused the low values of Redman, Willimot, and Wokes (16).

Throughout the small intestine below the duodenum the values with the high protein rations are consistently lower than those

TABLE IV

Effect of Excess Base on Intestinal Reaction with a High Protein Diet

Section of gastro-intestinal tract	Lactose added	Means	Mean deviation	Probable error
	<i>per cent</i>			
I*	None	6.43-6.38	-0.05	±0.03
	25	6.44-6.55	+0.11	±0.03
II*	None	6.85-6.80	-0.05	±0.04
	25	6.66-6.91	+0.25	±0.04
III*	None	7.34-7.26	-0.08	±0.05
	25	6.86-6.99	+0.13	±0.05
Cecum	None	7.28-7.06	-0.22	±0.04
	25	6.94-6.59	-0.35	±0.08
Colon	None	7.22-7.11	-0.11	±0.05
	25	7.09-7.00	-0.09	±0.05

* The small intestine was divided into three equal sections.

with the vegetable ration. Even in the large intestine the differences in the opposite direction are, on the basal diets, scarcely great enough to be significant. This is a rather interesting observation since an important element in the dietary control of intestinal reaction has been the assumption that a ration rich in animal protein favored the establishment of a proteolytic flora with a consequent alkaline environment as contrasted with the acid condition produced by a fermentative flora under the influence of a lactose-rich ration. The meat diet had 8.4 per cent fat as compared with 4.4 per cent in the other ration but the acidogenic properties of this could hardly be presumed to overcome the

effects of raising the protein content from 19.9 per cent to 47.5 per cent. In addition the vegetable ration contained more acid than either meat ration. Bollman and Mann (28), working with dogs, have reported findings similar to ours in this respect. It appears therefore that in the small intestine, animal protein may actually function as an acidogenic component of the intestinal contents, instead of the reverse.

Although the pH values for the large intestine are lower than those for the ileum they are still above the neutral point. This appears to be an unusual condition since the animals were receiving cod liver oil. Other workers have found the fecal reaction of rats to be acid under these conditions.

Effect of Lactose on Intestinal Reaction—Retger and Cheplin (3) found that 7 per cent of lactose brought about only a 50 per cent conversion of the flora into *Bacillus acidophilus*. Inouye (7) observed only slightly beneficial effects from 5 per cent lactose in the dietary prevention of tetany in parathyroidectomized dogs. Hence it might be predicted that 5 per cent lactose would make but little difference in the intestinal reaction. Such proved to be the case. With the meat diet, contrary to expectations, there was no significant change in any section. With the other ration the reactions of the cecum and colon were both reduced about 0.3 pH. The fact that these changes were in the region where, supposedly, absorption of calcium is at a minimum or does not take place at all reduces their significance from this standpoint.

On the other hand 25 per cent lactose in the ration effects an almost complete conversion of flora into an aciduric one and such a ration, to some extent at least, seems to protect parathyroidectomized dogs from tetany. It is apparent that at that level with the vegetable ration, the high content of lactose produced a marked reduction in pH throughout the length of the small intestine, cecum, and colon. With the high protein feeds on the other hand only when the effect was magnified by prolonged retention of material in the lower ileum and cecum were the values seriously affected. Even then the change was much less than with the other ration.

The changes in the reaction of the contents of the colon are particularly interesting because so much work has dealt with fecal reactions only. With the vegetable diet which, unsupplemented,

gave alkaline contents in the cecum and colon, there is a shift towards acidity with 5 per cent lactose and a more marked change with the larger amount of lactose. With the meat diets the changes, where significant in magnitude, were in the same direction, though smaller. It is apparent that the nature of the diet is a determining factor in increasing the fecal acidity by lactose feeding and it is possible that where an increase in fecal acidity has not been observed a diet similar to our meat ration in its influence on fecal acidity was in use.

Our results appear to contradict those of Cannon and McNease (13) who found a pronounced reduction in pH of the cecum and colon of rats on a diet of 1 part of lactose to 3 parts of uncooked beef. The explanation which offers itself at once is that the difference between their results and ours is due to the fact that their meat was raw and ours was cooked.

It may also be that the increased utilization of calcium which we observed with calves (6) and which Bergeim observed with rats (5) was apparent because of the use of vegetable diets capable of being influenced by lactose. Greenwald and Gross (9) and Gross (29) have both studied the effect of lactose on calcium and phosphorus retention. It seems that their results like Dragstedt's may have been complicated by the catharsis which the lactose produced. An acid stool may have resulted but the more rapid passage of food through the gut counteracted its effects. Inouye (7) found that increasing the casein content of the ration required a corresponding increase in lactose to prevent tetany. Dragstedt and Peacock (8) report better results in the prevention of tetany when a diet of bread and milk was used than with a ration of boiled rice, beef heart, and lactose which contained 13.5 per cent protein. In fact the surest way to induce tetany in dogs is to use a meat diet. There appear to be no data available on the utilization of calcium from a diet high in animal protein unless we accept, as indirect evidence, these results on tetany which indicate that it is not well utilized. Yet such a ration may be as acid, in the zone of active absorption, as a vegetable diet to which lactose has been added and from which presumably calcium is efficiently abstracted. In other words, intestinal acidity *per se* is not the determining factor in the absorption of calcium.

Neither is the mechanism of the action of lactose in enabling

the acidophilic organisms to overcome the proteolytic forms at all certain as Cannon and McNease (13) and Cruickshank (30) have pointed out. The latter showed that *in vitro* a reaction of below pH 5.0 was necessary to inhibit coliform bacteria. He noted in his subjects (infants) reactions no lower than pH 5.2 where the flora was predominantly aciduric. There is every reason to believe that there was the same change in flora in our animals which were receiving the large amounts of lactose yet the average values for the reaction of the contents of the large intestine were all over pH 6.0. Only two of forty-seven were below pH 5.0 and six were over pH 7.0. This in itself is strong indication that acidity is not the only factor determining the character of the intestinal flora.

Effect of Increased Dietary Base on Intestinal Reactions—Table IV shows the comparative results for the acid and alkaline diets. For the basal rations without lactose, the increased base content of the rations produced no result of significance except in the cecum where there was a *decrease* in the pH of the contents, small in magnitude but apparently significant. The same result was observed when 25 per cent of lactose was included in the diet although in the small intestine lactose apparently enabled the base to produce what should constitute its normal effect, an increase in pH. Further work leading to an explanation for these observations is now in progress.

SUMMARY

The pH of the intestinal contents of rats on a diet of grains and alfalfa increases to a maximum of about pH 8.0 in the lower ileum. There is a sharp drop to approximate neutrality at the ileocecal valve. With a meat diet the reaction reaches a maximum in the same region but, contrary to the usual assumption, the reaction of the whole small intestine is less alkaline than with the vegetable diet.

The ingestion of lactose amounting to 5 per cent of the ration slightly reduces the pH of the contents of the lower ileum and of the large intestine of rats on the vegetable diet but does not significantly affect the residue from a meat diet.

When the level of lactose is raised to 25 per cent there is a marked lowering of the pH of the contents of the whole digestive tract below the duodenum of rats fed the vegetable diet. With

the other ration the lowering is significant only in the ileum and cecum and even then is much smaller in magnitude than with the stock diet.

Increasing the base content of the high protein diet from 34 cc. of 0.1 N acid to 1369 cc. of 0.1 N alkali per 100 gm. caused a significant increase in alkalinity of the contents of the small intestine only when 25 per cent of lactose was included in the diet.

The effect on calcium absorption of the increased acidity under the influence of lactose feeding would appear to depend on the nature of the basal ration.

We wish to thank Professor S. E. Crowe for assistance in the statistical treatment of the results and Mr. C. C. Lightfoot for technical assistance in the preparation of samples.

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THE APPARENT DISSOCIATION CONSTANTS OF METHIONINE AND OF ISOSERINE*

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(Received for publication, May 25, 1931)

The present work is a continuation of the systematic survey of the apparent dissociation constants of the amino acids which has been in progress in this laboratory for some time (1). In this paper we are reporting the apparent dissociation constants of methionine and of isoserine.

Methionine was prepared synthetically according to a slight modification¹ of the procedure described by Windus and Marvel (2). The essential features of the modification are as follows: (a) In the condensation of malonic ester with chloroethylmethyl sulfide, the reagents are used in the following proportions: 1 mol of chloroethylmethyl sulfide, 1 mol of sodium, and 1.5 mols of malonic ester. It was found possible to increase the yield of methylthioethylmalonic ester from 45 per cent to 67 per cent of the theoretical. (b) Instead of heating the hydrobromide of methylthioethylaminomalonic acid in the dry state for purposes of decarboxylation, it was found advantageous to suspend the compound in xylene and to heat it with mechanical stirring at the boiling point of that liquid. Charring is practically eliminated and the yield of methionine is somewhat increased. The preparation of methionine was recrystallized several times from water and dried over phosphorus pentoxide. It gave within experimental

* Aided by a grant from The Chemical Foundation, Incorporated, and the Research Board of the University of California. We are indebted to the Cyrus M. Warren Fund of the American Academy of Arts and Sciences for the loan of the type K potentiometer.

¹ Dr. C. S. Marvel has made similar modifications in his published experimental procedure. Private communication.

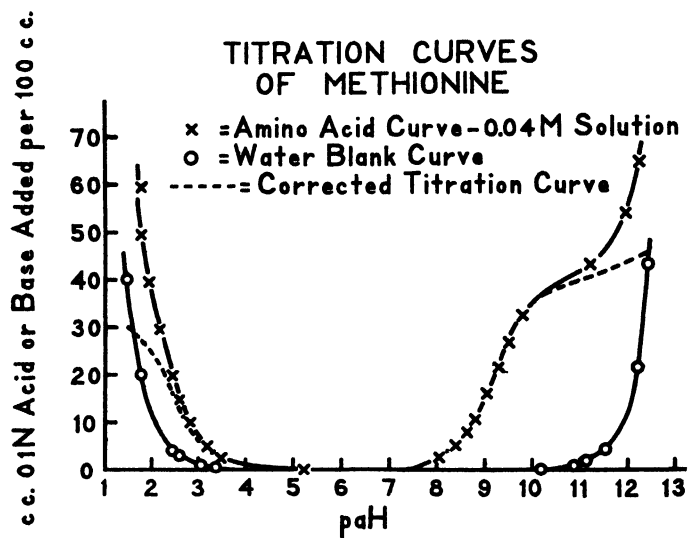


FIG. 1

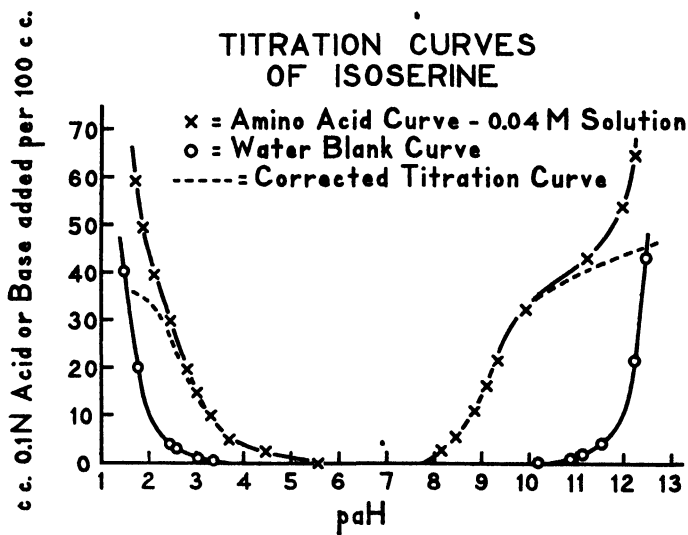


FIG. 2

limits theoretical nitrogen values. Isoserine was synthesized according to the procedure of Fischer and Leuchs (3). It was likewise dried over phosphorus pentoxide and yielded theoretical nitrogen values.

Estimations of hydrogen ion activity in the amino acid solutions were carried out at 25° with the aid of the quinhydrone and hydrogen electrodes in a manner previously described in work from this laboratory (4).

The titration curve of methionine is shown in Fig. 1 and that of isoserine in Fig. 2. For methionine the following values for the apparent dissociation constants at 25° were calculated: $K'_a = 6.17 \times 10^{-10}$, $pK'_a = 9.21$, $K'_b = 1.91 \times 10^{-12}$, $pK'_b = 11.72$, and $pI = 5.74$. The values for isoserine at this temperature are: $K'_a = 5.37 \times 10^{-10}$, $pK'_a = 9.27$, $K'_b = 6.03 \times 10^{-12}$, $pK'_b = 11.22$, and $pI = 6.02$. The dissociation constants of methionine are characteristic of the monoaminomonocarboxylic amino acids. The value for K'_a of methionine is, however, slightly greater than that for most of the members of this group of amino acids. It is nearly the same as that of phenylalanine. The value for the apparent acid dissociation constant of the latter amino acid is slightly greater than the value of the corresponding constant of alanine. This is probably due to the presence of the phenyl ring in the molecule. The fact that the value for the apparent acid dissociation constant of methionine is somewhat greater than that of either alanine or norleucine may possibly be due to the presence in methionine of the methylthiol group. Some indications of the effect of the presence of an ether group in the molecule are obtained by comparing the dissociation constants of methoxyacetic acid and of acetic acid. At 25° the dissociation constant for methoxyacetic acid is 3.4×10^{-4} while that for acetic acid is 1.86×10^{-5} (5). The presence of the methoxy group in the molecule increases the value for the dissociation constant. The apparent basic dissociation constant of methionine is slightly less than that of phenylalanine and very nearly the same as that of alanine and of norleucine.

A comparison of the dissociation constants of isoserine and of serine is also of interest. The apparent basic dissociation constant of isoserine is somewhat greater than the corresponding value for serine. This is in line with other work reported from this labora-

tory on the effect of the position of the amino group on the value for the apparent basic dissociation constant (6). The effect of the shift of the amino group as in isoleucine is not as marked as in the case of β -alanine. This is probably due to a shifting of the hydroxy group to the α position. A somewhat similar conclusion can be drawn from Chibnall and Cannan's (7) work on the hydroxy-asparagines. The apparent acid dissociation constant of isoleucine is a little less than that of serine.

SUMMARY

The apparent acid and basic dissociation constants of methionine and of isoleucine have been determined. Comparisons with certain other amino acids and related compounds have been made.

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THE APPARENT DISSOCIATION CONSTANTS OF HYDROXYVALINE*

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(Received for publication, May 25, 1931)

The presence of hydroxyvaline as a constituent of oat protein was first shown by Schryver and Buston (1). It was later isolated from zein by Brazier (2). The position of the hydroxy group in the molecule has not been established by synthesis. From analogy to other hydroxyamino acids the hydroxy group of hydroxyvaline is probably in the beta position. The amino acid has interested us in that it affords another opportunity of determining the influence of the hydroxy group on the apparent acid and basic dissociation constants of amino acids.

Hydroxyvaline was isolated from oat protein essentially according to the method described by Brazier (2). It was recrystallized several times from water-acetone mixtures and finally dried over phosphorus pentoxide. It yielded theoretical nitrogen values. The technique used in preparing the titration curve and the method employed in determining the dissociation constants are the same as described in the preceding paper. The titration curve is shown in Fig. 1. The following values were obtained for the apparent dissociation constants at 25°: $K'_a = 1.97 \times 10^{-10}$, $pK'_a = 9.71$, $K'_b = 4.05 \times 10^{-12}$, $pK'_b = 11.39$, and $pI = 6.15$. These values do not differ very markedly from those which have been reported for valine. The apparent basic dissociation constant of hydroxyvaline is a little greater than that of valine. The effect is, however, not very pronounced. Chibnall and Cannan found that the basic

* Aided by a grant from The Chemical Foundation, Incorporated, and the Research Board of the University of California. We are indebted to the Cyrus M. Warren Fund of the American Academy of Arts and Sciences for the loan of the type K potentiometer.

dissociation constant of β -hydroxyasparagine was a little greater than that of β -asparagine (3). In the case of β -hydroxyglutamic acid (4) a tendency in the same direction is noted. The apparent acid dissociation constants are also increased slightly by the presence of the hydroxy group in the latter amino acid. The presence of the hydroxy group in serine appears to augment the value of the apparent acid dissociation constant somewhat. This may possibly be due to its being in the terminal position.

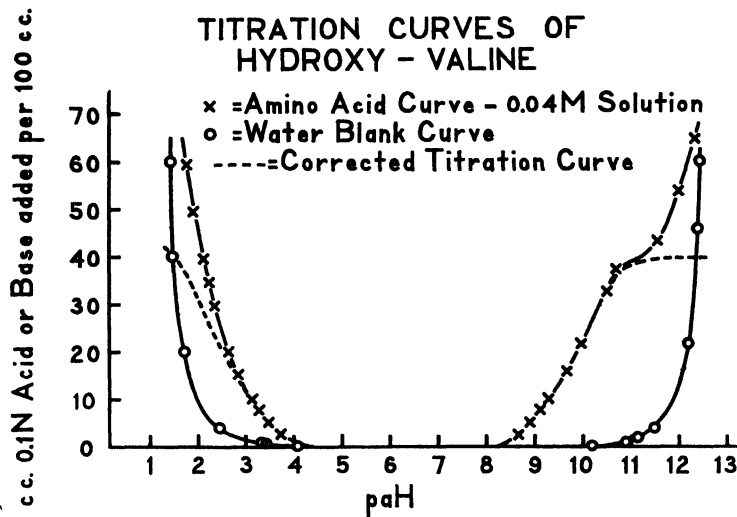


FIG. 1

SUMMARY

The apparent acid and basic dissociation constants of hydroxyvaline have been determined. Comparisons have been made between the apparent dissociation constants of hydroxyvaline and other hydroxyamino acids.

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CONFIGURATIONAL RELATIONSHIP OF HYDROCARBONS

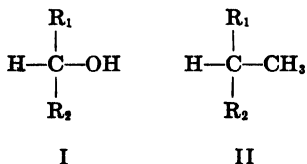
III. THE OPTICAL ROTATIONS OF THE HYDROCARBONS OF THE SERIES METHYLISOBUTYLMETHANE

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(Received for publication, April 28, 1931)

Previous observations in this laboratory¹ have led to the conclusion that in simple substances of the types I and II, the direction of rotation



is determined by the respective weights of the groups R_1 and R_2 . When R_1 is smaller in weight than R_2 , the substances of type I rotate to the right. If R_1 contains a polar group, however, the direction of rotation is determined by the distance of the polar group from the asymmetric carbon atom and not by the weights of the radicles, unless the polar group is very far removed from the asymmetric carbon atom.

Later, on the basis of indirect evidence, it was concluded that when R_1 contains an isopropyl group, then again the direction of rotation of a substance is determined by the distance of the isopropyl group from the asymmetric carbon atom and unless the isopropyl group is very far removed from the asymmetric carbon atom, the weights of the other radicles play a subordinate part in their effect on the direction of rotation.

On the basis of these considerations, and on the basis of the

¹ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 405, 761 (1931).

observations on the rotations of the secondary carbinols of the isobutyl series, it was expected that configurationally related hydrocarbons of the homologous series

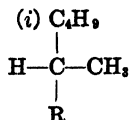


TABLE I*

Maximum Molecular Rotations of Configurationally Related Hydrocarbons Calculated on Basis of Maximum Rotation of Acids from Which They Were Prepared

$[\text{M}]_D^{25}$

	$\begin{array}{c} \text{---CH}_2\text{COOH}\dagger \\ (1) \end{array}$	$\begin{array}{c} \text{---C}_2\text{H}_5 \\ (2) \end{array}$	$\begin{array}{c} \text{---}n\text{-C}_3\text{H}_7 \\ (3) \end{array}$	$\begin{array}{c} \text{---}n\text{-C}_4\text{H}_9 \\ (4) \end{array}$	$\begin{array}{c} \text{---}n\text{-C}_6\text{H}_{11} \\ (5) \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \diagup \\ \text{CH} \\ \diagdown \\ \text{CH}_3 \\ \text{---CH}_2\text{---} \\ (6) \end{array}$
$\begin{array}{c} \text{C}_2\text{H}_5\text{---CH---} \\ \\ \text{CH}_3 \end{array}$	+10.35	0	+9.9	+11.4	+12.0	+21.3
$\begin{array}{c} n\text{-C}_3\text{H}_7\text{---CH---} \\ \\ \text{CH}_3 \end{array}$	-3.60	-9.9	0	+1.7	+2.4	+14.9
$\begin{array}{c} n\text{-C}_4\text{H}_9\text{---CH---} \\ \\ \text{CH}_3 \end{array}$	-6.06	-11.4	-1.5	0	+0.8	+11.9
$\begin{array}{c} n\text{-C}_6\text{H}_{11}\text{---CH---} \\ \\ \text{CH}_3 \end{array}$	-8.12	-12.5	-2.4	-0.8	0	+9.3

* On reexamination it was found that the methylethyl-*n*-butyl and methylethyl-*n*-amylmethanes previously described contained traces of the unsaturated hydrocarbons. The repurified methylethyl-*n*-amylmethane was found to have the rotation given in this table. The methyl-*n*-butylpropionic and methyl-*n*-amylpropionic acids were then resolved to maximum and converted into the hydrocarbons. Therefore, the values given in this *Journal* (Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 764 (1931)) should be corrected according to this table.

† See Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 761 (1931).

would all rotate in the same direction regardless of the molecular weight of the radicle R; furthermore, it was expected that the numerical values of the rotation of the individual members would be in descending order, the member with R equals C_2H_5 having the highest rotation.

A series of hydrocarbons of this general type has now been prepared. In Table I, Column 6, are summarized the results of observations on the hydrocarbons of this series. In Columns 2 to 5 are given the rotations of the hydrocarbons of the normal series. In the latter the rotations of the carbinols situated above the sym-

TABLE II
Experimental Values for Molecular Rotations of Configurationally Related Hydrocarbons Containing an Isobutyl Group

	$-CH_2COOH$	$-CH=C \begin{matrix} CH_3 \\ CH_3 \end{matrix}$	$-CH_2-CH \begin{matrix} CH_3 \\ CH_3 \end{matrix}$
$C_2H_5-CH-CH_3$	-6.0		-12.4
$n-C_3H_7-CH-CH_3$	+1.1	-7.7	-4.5
$n-C_4H_9-CH-CH_3$	+1.8	-5.0	-3.6
$n-C_6H_{11}-CH-CH_3$	+2.5	-4.8	-2.8

metric member rotate in opposite direction from those below. The direction of rotation of the members of the isobutyl series does not change and is of descending order, as was expected. Thus, the direction of rotation of simple substances can in a way be predicted on the basis of earlier observations.

The configurational relationships of the hydrocarbons here discussed have been established on the basis of the method of preparation. They have been prepared from disubstituted 1,1-propionic acid (3) of known configurational relationship.

The maximum values given in Table I are derived by computation on the basis of the knowledge of the maximum rotation of the disubstituted acids.

The experimental values for molecular rotations of configurationally related hydrocarbons containing an isobutyl group are given in Table II.

EXPERIMENTAL

2,4-Dimethyl Heptanol-2—To 1 mol of methyl magnesium iodide in ether were added 60 gm. of ethyl ester of 2-propyl butyric acid-4 (from 2-propyl butyric acid-4, $[M]_D^{24} = +1.08^\circ$). The Grignard reagent was decomposed in the usual manner and the carbinol distilled. B.p. $134-136^\circ$ at 760 mm., yield 33 gm.

Levo-2,4-Dimethyl Heptane—33 gm. of 2,4-dimethyl heptanol-2 (above) were mixed with 10 gm. of oxalic acid and distilled. Dehydration took place very readily. The unsaturated hydrocarbon which collected in the receiver was separated from the water, dried with sodium sulfate, then distilled from a small piece of metallic sodium. B.p. $134-136^\circ$ at 760 mm., yield 25 gm., $D_4^{24} = 0.739$.

$$[\alpha]_D^{24} = \frac{-4.51^\circ}{1 \times 0.739} = -6.10^\circ. \quad [M]_D^{24} = -7.69^\circ \text{ (homogeneous)}$$

3.562 mg. substance: 11.045 mg. CO_2 and 4.540 mg. H_2O .

C_9H_{18} . Calculated. C 85.62, H 14.38

Found. " 84.55, " 14.26

The unsaturated hydrocarbon was mixed with 0.5 gm. of platonic oxide catalyst and reduced by shaking with hydrogen under a pressure of 30 pounds per square inch. The reduction took only 15 minutes for completion. The hydrocarbon was separated from the catalyst and shaken with cold concentrated sulfuric acid. It was washed with sodium carbonate solution, followed by water, and dried with dry sodium sulfate. It was then distilled from a small piece of metallic sodium. B.p. $131-131.5^\circ$ at 760 mm., yield 19 gm., $D_4^{22} = 0.733$.

$$[\alpha]_D^{22} = \frac{-2.57^\circ}{1 \times 0.733} = -3.51^\circ. \quad [M]_D^{22} = -4.49^\circ \text{ (homogeneous)}$$

5.521 mg. substance: 17.140 mg. CO₂ and 7.585 mg. H₂O.

C₉H₂₀. Calculated. C 84.27, H 15.73

Found. " 84.65, " 15.39

2,4-Dimethyl Octanol-2—This was prepared from 1 mol of methyl magnesium iodide and 60 gm. of ethyl ester of 2-butyl butyric acid-4 (from 2-butyl butyric acid-4, $[M]_D^{24} = +1.83^\circ$). The carbinol was isolated in the usual way. Due to its instability it was distilled, but not further purified.

Levo-2,4-Dimethyl Octane—The carbinol from the above preparation was mixed with 10 gm. of oxalic acid and distilled. The unsaturated hydrocarbon was separated from the water, dried with dry sodium sulfate, then distilled from a small piece of metallic sodium. B.p. 62° at 30 mm., yield 27 gm., $D_4^{24} = 0.743$.

$$[\alpha]_D^{24} = \frac{-2.67^\circ}{1 \times 0.743} = -3.59^\circ. \quad [M]_D^{24} = -5.04^\circ \text{ (homogeneous)}$$

4.150 mg. substance: 13.030 mg. CO₂ and 5.370 mg. H₂O.

C₁₀H₂₀. Calculated. C 85.61, H 14.39

Found. " 85.61, " 14.53

20 gm. of the unsaturated hydrocarbon were mixed with 0.5 gm. of platonic oxide and reduced by hydrogen under a pressure of 30 pounds per square inch. The hydrocarbon was purified as described for 2,4-dimethyl heptane. B.p. 70° at 40 mm., yield 17 gm., $D_4^{24} = 0.725$.

$$[\alpha]_D^{24} = \frac{-1.82^\circ}{1 \times 0.725} = -2.51^\circ. \quad [M]_D^{24} = -3.57^\circ \text{ (homogeneous)}$$

4.150 mg. substance: 12.870 mg. CO₂ and 5.790 mg. H₂O.

C₁₀H₂₂. Calculated. C 84.40, H 15.60

Found. " 84.56, " 15.61

2,4-Dimethyl Nonanol-2—This was prepared from 1 mol of methyl magnesium iodide and 60 gm. of ethyl ester of 2-amyl butyric acid-4 (from 2-amyl butyric acid-4, $[M]_D^{24} = +2.47^\circ$). The carbinol was isolated and distilled as previously described but not purified further.

Levo-2,4-Dimethyl Nonane—The carbinol above was mixed with 10 gm. of oxalic acid and distilled. The unsaturated hydrocarbon was separated from the water, dried with dry sodium sulfate, and distilled from metallic sodium. B.p. 79° at 30 mm., $D_4^{24} = 0.751$.

$$[\alpha]_D^{24} = \frac{-2.32^\circ}{1 \times 0.751} = -3.09^\circ. \quad [M]_D^{24} = -4.76^\circ \text{ (homogeneous)}$$

3.935 mg. substance: 12.295 mg. CO₂ and 5.040 mg. H₂O.

C₁₁H₂₂. Calculated. C 85.62, H 14.38

Found. " 85.20, " 14.33

20 gm. of the unsaturated hydrocarbon were mixed with 0.5 gm. of platonic oxide and reduced by hydrogen under a pressure of 30 pounds per square inch. The hydrocarbon was isolated and purified as described for 2,4-dimethyl heptane. B.p. 75° at 25 mm., yield 18 gm., $D_4^{24} = 0.731$.

$$[\alpha]_D^{24} = \frac{-1.33^\circ}{1 \times 0.731} = -1.82^\circ. \quad [M]_D^{24} = -2.84^\circ \text{ (homogeneous)}$$

3.812 mg. substance: 11.835 mg. CO₂ and 5.270 mg. H₂O.

C₁₁H₂₄. Calculated. C 84.51, H 15.49

Found. " 84.66, " 15.47

PYRROLE AS A CATALYST FOR CERTAIN BIOLOGICAL OXIDATIONS

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Except for some recent work on the pharmacology of pyrrole by Biglioli (1) and Rabbeno (2), little work has been done on the action of this compound with biological systems. Shimizu (3) showed that when it was injected into rabbits, it was methylated and excreted in the urine. Introzzi (4) injected pyrrole subcutaneously and produced a pigmentation of the skin and hair after exposure to light. Saccardi (5) and Comini (6) claim that a pigment approximating to a true melanin can be formed in the skin of many animals after the administration of pyrrole. Finally Rondoni (7) showed that incubation of pyrrole with the melanogenic organs of the cuttlefish produced a melanin, and similarly incubation with mammalian tissue, especially liver, produced a dark pigment. It is this last observation that we have investigated more closely with regard to the possible oxidations involved.

When pyrrole is added to liver the oxygen uptake of the pyrrole and liver is only 20 to 30 c.mm. greater than that of the liver alone after 5 or 6 hours. If, however, the liver is washed two or three times with buffer, the addition of pyrrole causes an appreciable extra oxygen uptake. This uptake is practically independent of the concentration of pyrrole but is dependent on the concentration of liver. This extra oxygen uptake is further increased by the addition of lactic acid to the mixture. Lactic acid and washed liver in contradistinction to lactic acid and unwashed liver take up little or no oxygen, the amount depending on the thoroughness of the washing. If pyrrole is added to a mixture of lactic acid and washed liver, oxygen is taken up in an amount exactly equal to the

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quantitative oxidation of lactic acid to pyruvic acid. Within limits the amount of pyrrole present has no influence on the oxygen uptake. Too much pyrrole slows the reaction because of its toxic effects.

It is obvious that pyrrole itself is not being oxidized, but is acting as a hydrogen transport system for certain unknown substances in the liver and for lactic acid. It has been shown by Michaelis and Salomon (8) that liver naturally contains such a system which can be extracted by washing and when added to red blood cells will cause them to oxidize lactic acid. This explains why unwashed liver will oxidize lactic acid and washed will not; and why the addition of pyrrole to unwashed liver has so little effect, for it has to compete with a hydrogen transport system already there. It is only when this has been washed away that the effect of the pyrrole can be seen. The mechanism by which pyrrole acts will be discussed below.

A dark color is always seen after the liver has been incubated with pyrrole, confirming the observations of Rondoni. But this is a by-product and has nothing to do with the oxygen uptake, for certain livers, such as rabbit liver, will not take up any oxygen in the presence of pyrrole but still form this melanin-like substance.

EXPERIMENTAL

The liver of the white rat was used in all the following experiments. The fresh liver was chopped as finely as possible with scissors. It was then ground in a mortar with 20 cc. of 0.05 M phosphate buffer, pH 7.3, and centrifuged. This process was repeated three times, after which the washings were free of hemoglobin. It was then ground again with a buffer containing 2 per cent sodium fluoride to prevent bacterial growth and the formation of lactic acid from any remaining glycogen or glucose. This mixture was then squeezed through muslin and the resulting homogeneous liver suspension used.

This preparation had a deep red color which was due to the pigment fixed to the protein of the cell, for all the free hemoglobin had been washed away. Occasionally, however, the liver suspension had only a very pale color, in which case it was always inactive. The fixed pigment is therefore necessary for the reaction with pyrrole to take place.

The suspension was then suitably diluted, for if it is too thick the substance in the liver which is activated by pyrrole is present in such a large excess that the addition of lactic acid has little effect. It is possible to get a dilution which with pyrrole alone will take up only 10 to 20 c.mm. of oxygen and is still strong enough to oxidize the lactic acid quantitatively to pyruvic acid.

The pyrrole was a synthetic product from the Eastman Kodak Company. It was redistilled and kept at 0° and the solution made up freshly for each experiment by adding 0.1 cc. (94 mg.) to 9.9 cc. of buffer. This amount dissolves completely. From 0.1 to 0.4 cc. of this solution was used in the experiments. The lactic

TABLE I
Experiment 1,a

Measurements were made at a temperature of 38°.

Time	Oxygen uptake					
	Vessel 1	Vessel 2	Vessel 2 minus Vessel 1	Vessel 3	Vessel 4	Vessel 4 minus Vessel 3
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
1 hr.	16	21	5			
2 hrs.	33	46	13	18	17	-1
3 "	41	71	30	20	29	9
4 "	58	104	46	27	44	17
4 " 45 min.	68	117	49	30	52	22
5 " 35 "	80	130	50	39	61	22
6 " 5 "	85	136	51	42	65	23

acid was boiled to insure complete hydrolysis of the anhydride and then neutralized by NaOH, and the concentration estimated by the Friedemann, Cotonio, and Shaffer method (9) as modified by Davenport and Davenport (10).

The Barcroft-Warburg apparatus was used to measure the oxygen uptake at temperatures of 28° or 38°.

Experiment 1,a shows that the oxygen uptake of the washed liver alone is increased by the addition of pyrrole and that a definite amount of some substance in the liver is being oxidized. What this substance is, is not known, but it is likely to be some group in the protein molecule or possibly some lipid or unsaturated fat for

all the water-soluble substances in the liver have been washed out. This point is being investigated further.

Experiment 1,a

Vessel 1 contained 0.5 cc. of washed liver suspension and 1.5 cc. of buffer; Vessel 2 contained 0.5 cc. of liver, 1.3 cc. of buffer, and 0.2 cc. of pyrrole solution (1.9 mg.). Vessels 3 and 4 were like Vessels 1 and 2 respectively except that the liver suspension was diluted three times. The figures given in Table I represent oxygen uptake in c.mm. at a temperature of 38°.

TABLE II

Experiment 1,b

Measurements were made at a temperature of 38°.

Time	Oxygen uptake				
	Vessel 1	Vessel 2	Vessel 3	Vessel 2 minus Vessel 1	Vessel 3 minus Vessel 1
	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.
35 min.	16	20	31	4	15
1 hr., 5 min.	28	38	44	10	16
1 " 35 "	33	56	60	23	27
2 hrs., 25 "	45	97	101	52	56
3 " 20 "	54	130	134	76	80
4 " 15 "	64	162	157	98	93
5 " 20 "	82	194	187	112	105
6 " 5 "	94	214	206	120	112

It will be seen that the oxygen uptake is, within experimental error, proportional to the amount of liver present. Moreover, after 5 hours the oxygen uptake due to the presence of pyrrole ceases, thus indicating that a definite amount of some substances in the liver is being oxidized. That pyrrole itself is not being oxidized is shown by the following experiment.

Experiment 1,b

Vessel 1 contained 0.5 cc. of washed liver suspension and 1.5 cc. of buffer; Vessel 2 contained 0.5 cc. of liver, 1.3 cc. of buffer, and 0.2 cc. of pyrrole solution (1.9 mg.); Vessel 3 contained 0.5 cc. of

liver, 1.1 cc. of buffer, and 0.4 cc. of pyrrole solution (3.8 mg.). The liver preparation was more concentrated than that used in Experiment 1,a and the temperature was 38° (Table II).

After 6 hours the uptake due to 0.2 cc. of pyrrole is 120 c.mm., and that due to 0.4 cc. of pyrrole 112 c.mm. The amounts of oxygen uptake with the two concentrations are within a few c.mm. of one another. This difference is within the experimental error. Experiments 1,a and 1,b thus prove definitely that the pyrrole itself is not oxidized, but that it catalyzes the oxidation of some substances present in the liver.

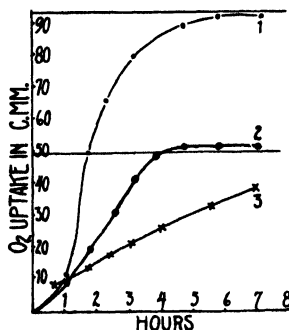


FIG. 1. Curve 1 shows the O₂ uptake of the liver suspension + 0.75 mg. of lactic acid and 1.9 mg. of pyrrole minus the O₂ uptake of the liver and lactic acid alone. Curve 2 shows the same with 0.375 mg. of lactic acid. Curve 3 shows the O₂ uptake of liver + 0.75 mg. of lactic acid minus the O₂ uptake of the liver alone. The horizontal lines represent the theoretical uptake for the oxidation of 0.375 mg. and 0.75 mg. of lactic acid to pyruvic acid.

In trying the effect of lactic acid it was necessary to adjust conditions so that the oxygen uptake due to the liver and pyrrole alone was as small as possible and yet that there was present enough to insure the oxidation of the lactic acid. Within limits these conditions were easily obtained, for the pyrrole seemed to act preferentially as a catalyst for the oxidation of the lactic acid, as was shown by the exactly theoretical values obtained for the oxidation of the lactic to pyruvic acid without the small oxygen uptake of the liver and pyrrole alone being taken into account. The justification of this assumption is that the concentration of lactic acid alone determined the oxygen uptake, regardless of the

concentration of pyrrole and the concentration of liver, unless the liver was there in too large amounts.

Fig. 1 shows the oxygen uptake with liver and pyrrole with two concentrations of lactic acid. It will be seen that the theoretical value for the oxidation of lactic acid to pyruvic acid is obtained without taking into account the uptake of the liver and pyrrole which in this case was between 20 and 30 c.mm. This reaction presumably does not take place in the presence of lactic acid for the theoretical uptake has been obtained when the uptake due to the pyrrole and liver has been zero or as much as 50 c.mm. If it is more than 50 c.mm. then the system in the liver competes with the lactic acid system and an indeterminate result is obtained.

The theoretical uptake is obtained by subtracting the uptake of liver and lactic acid from that of the liver lactic acid and pyrrole. In a well washed liver preparation the oxygen uptake of liver and lactic acid is only slightly greater than that of liver alone. This slight increase is not due to the oxidation of the lactic acid, for it is independent of the lactic acid concentration, and the curve (see Fig. 1) obtained by subtracting the uptake of liver alone from liver plus lactic acid is a straight line; *i.e.*, it is not a biological oxidation in which the substrate is the limiting factor. It might best be termed a specific dynamic effect. It amounts ordinarily to 20 or 30 c.mm.

The oxidation does not start immediately on mixing the pyrrole with the liver and lactic acid. There is a latent period, varying with the temperature, of about 2 hours at 28° and 1 hour at 38°. This gives the curves an S shape. The significance of this will be discussed below.

As in the case of liver alone, the concentration of pyrrole makes no difference to the oxygen uptake. In fact, the lactic acid system is a little more sensitive to the toxic effects of a high concentration of pyrrole than the liver alone. Whereas with both 0.1 cc. and 0.2 cc. of pyrrole solution there is very little difference in the rate at which the lactic acid is oxidized, with 0.3 cc. there is a marked slowing. Thus after 4 hours with 0.2 cc. there is an oxygen uptake of 89 c.mm. (100 c.mm. are the theoretical uptake), whereas with 0.3 cc. only 68 c.mm. have been taken up. Less than 0.1 cc. of pyrrole also shows a slowing due to the fact that not enough pyrrole is available.

Both KCN and pyrophosphate completely inhibit the oxidations catalyzed by the pyrrole with liver alone and liver and lactic acid. $\frac{1}{200}$ N KCN was used, and whereas the control had taken up the theoretical amount of oxygen in 6 hours, with KCN only 20 c.mm. had been taken up. These 20 c.mm. were taken up toward the end as the KCN concentration diminished due to distillation into the soda. The same effect was noted with pyrophosphate. 0.1 N pyrophosphate buffer, pH 7.3, was used instead of ordinary phosphate buffer, and there was a complete inhibition for 4 hours, after which, due probably to the hydrolysis of the pyrophosphate, an oxygen uptake began.

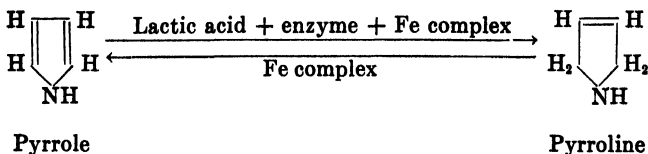
The liver of the white rat is the most satisfactory tissue for studying the effects of pyrrole. Guinea pig and cat livers react slowly and rabbit livers not at all. It is difficult to obtain active preparations with other tissues.

Red blood cells were also tried. It was shown by Harrop and Barron (11) that methylene blue will enable these cells to oxidize lactic acid to pyruvic acid. Later Warburg, Kubowitz, and Christian (12) and Wendel (13) showed that this was due to the formation of methemoglobin by the methylene blue. Evidently pyrrole is unable to do this, for red blood cells will not oxidize lactic acid in its presence.

DISCUSSION

It is obvious that the action of pyrrole is very like that of methylene blue in restoring the oxygen uptake to washed tissue. It is well known that washed muscle and lactic acid take up no oxygen, but the addition of methylene blue causes a quantitative oxygen uptake. The lactic acid enzyme system reduces the methylene blue to methylene white and the latter is then reoxidized by oxygen to methylene blue again. This last reaction occurs spontaneously, requiring no activation on the part of the tissue. The oxygen uptake catalyzed by methylene blue is not inhibited by KCN or pyrophosphate. By analogy it would be possible to postulate the reduction of pyrrole to pyrroline and the reoxidation of the pyrroline as in the following diagram. The only difference from methylene blue is that the reduction or reoxidation does not appear to take place spontaneously, but requires activation by some iron complex, as shown by the fact that KCN

and pyrophosphate inhibit it. This iron complex may be necessary for the reduction of pyrrole, the oxidation of pyrroline, or both.



This theory, however, does not explain the latent period before the oxidation begins. This latent period seems to indicate that it is not the pyrrole itself but perhaps some condensation product which acts in the above manner. Whatever the details of the mechanism, this general scheme is correct, as shown by the fact that the oxygen uptake is not a function of the concentration of pyrrole but only of the concentration of the substrate. The pyrrole system is not specific for lactic acid, for citric acid is also oxidized by washed liver in its presence. But due to the fact that it is not known to what citric acid goes, it is difficult to study this reaction quantitatively.

SUMMARY

1. Pyrrole added to washed liver cells enables these cells to oxidize lactic and citric acids. It itself is not oxidized.

2. In its presence lactic acid is quantitatively oxidized to pyruvic acid. KCN and pyrophosphate inhibit this oxidation completely.

3. The action of pyrrole is compared with that of methylene blue in restoring the oxygen uptake of washed tissue in the presence of substrates.

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THE EFFECT OF IRON AND CYANIDES ON THE SPONTANEOUS OXIDATION OF DIALURIC ACID*

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INTRODUCTION

The catalytic action of iron in oxidations has been known for many years, and since iron is universally present in cells, physiologists and biochemists from very early times have almost universally ascribed to it an important rôle in oxidation. Professor J. U. Lloyd having suggested the study of iron in cell respiration, this investigation was begun under the direction and plan of Professor A. P. Mathews. Mathews and Walker (1) showed how greatly iron catalyzed the spontaneous oxidation of cysteine and more recently Warburg and Keilin have ascribed to it and its compound, cytochrome, the rôle of the respiratory enzyme. Warburg and Sakuma (2) believed that the whole of the oxidation of cysteine in air was due to iron and that cysteine entirely free from iron was incapable of autoxidation. Harrison (3), however, observing with even very pure cysteine some autoxidation, left the question open. Recently in this laboratory, Gerwe (4), in another investigation in this same series, demonstrated that cysteine is spontaneously oxidizable, although at a very slow rate, and iron greatly catalyzes the oxidation. Gerwe also found that cyanide inhibited the catalytic effect of iron, but not the spontaneous oxidation of cysteine itself.

This work was begun with the view of studying the respiratory

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function of the cell nucleus. The nucleus has always been regarded as important in cell respiration since cells without nuclei respire at a very slow rate, even though they may contain glutathione and respiratory iron compounds (red blood corpuscles of mammals). Among the pyrimidine compounds of the nucleus, dialuric acid oxidizes at a very rapid rate, passing over into alloxan and alloxantin. This oxidation is being studied in this laboratory, and the relation of the oxidation to the reaction of the medium has already been published (5).

The present paper shows (1) that dialuric acid free from iron oxidizes spontaneously at a very rapid rate; (2) that this oxidation is at a maximum at pH 7.0 to 7.4 and falls rapidly on each side of the optimum to a minimum at pH 6.8 and 7.6; (3) that iron catalyzes the reaction on either side of the optimum, but not noticeably at the optimum, possibly because oxygen could not enter the solution fast enough; and (4) that cyanides have no effect on the spontaneous oxidation, but inhibit only the catalytic action of iron.

In a previous paper, the author (5) has described the spontaneous oxidation of dialuric acid, a pyrimidine and an oxidation product of uric acid. This oxidation was found to be extremely sensitive to the reaction of the medium, going only with any considerable speed within the limits of neutrality. In this susceptibility and the position of the optimum, dialuric acid oxidation resembles that of cysteine and also closely parallels the condition in living cells where the oxidations are also extremely sensitive to such variations and in which there is the same optimum of oxidation at this alkalinity. It is the purpose of the present investigation to discover: (1) whether this reaction is accelerated by iron; (2) whether dialuric acid is truly autoxidizable in the absence of iron (small amounts were present in the previous work); and (3) whether the oxidation is interfered with by cyanides.

The effect of iron on the oxidation of another member of the pyrimidine family, thymine, has already been reported. Johnson and Baudisch (6) observed that thymine is readily oxidized by FeSO_4 , NaHCO_3 , and air, yielding the products urea, acetol, and pyruvic acid. Baudisch and Bass (7) studied the action of H_2O_2 , and of H_2O_2 and FeSO_4 on thymine. They found the same products were formed as in the oxidation with FeSO_4 , NaHCO_3 , and

air. Bass (8) demonstrated that under the influence of ultra-violet light oxygen was capable of attacking the thymine molecule in the presence of FeSO_4 , so that subsequent hydrolysis gave urea and pyruvic acid.

I. Action of Iron

Method

Dialuric acid was prepared according to the directions in the preceding paper (5) with various modifications in the final step of the process to insure freedom from iron. Crystallizations were carried out in fused quartz vessels until the product was free from iron. A 2 gm. sample of the final product, ignited in a quartz crucible and tested for iron, did not give the slightest trace of color when tested by the thiocyanate method described by Yoe (9). This method will detect 0.000,000,1 gm. of iron. The water, hydrochloric acid, and ammonia used throughout the experiments were distilled in quartz and samples of these solutions, in 500 cc. quantities, were evaporated in quartz dishes and tested for iron. In no case was a trace of color obtained in the KCNS test.

The Barcroft respirometer was used, as before, for the measurement of the amount of oxygen absorbed by the oxidation of the dialuric acid. The experiments were made in the same manner. A 10 mg. sample of the dialuric acid was dissolved in iron-free distilled water, neutralized to the desired pH with iron-free ammonia, and buffered with a buffer solution of the same pH. The buffer solutions used were combinations of varying amounts of 0.2 M KH_2PO_4 and 0.2 M NaOH, according to Clark (10), and gave values ranging from pH 6.0 to pH 8.0. The buffer solutions were essentially free from iron. 500 cc. quantities were analyzed and found to contain 0.000,000,5 gm. of iron. 50 cc. quantities gave no test, showing that they contained less than $\frac{1}{10,000,000}$ gm. of iron, which is the sensitivity limit of the test. The 5 cc. of buffer solution used in each experiment could not have contained therefore, more than $\frac{5}{1,000,000,000}$ gm. of iron.

A series of experiments was carried out in which the oxidation of

TABLE I
Effect of Small Amounts of Iron

The figures are for absorption, measured as mm. difference of manometer level.

pH.....	6.0	6.0	6.0	6.4	6.4	6.4	6.4	6.4	6.8	6.8	6.8	7.0	7.0	7.0	7.0
	Dialuric acid alone	+ 0.0001 mg. Fe	+ 0.001 mg. Fe	+ 0.005 mg. Fe	+ 0.001 mg. Fe	+ 0.001 mg. Fe	+ 0.001 mg. Fe	+ 0.005 mg. Fe	Dialuric acid alone	+ 0.0001 mg. Fe	+ 0.001 mg. Fe	Dialuric acid alone	+ 0.0001 mg. Fe	+ 0.001 mg. Fe	+ 0.005 mg. Fe
Time shaken															
min.															
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	51	76	84	24	34	46	83	16	54	28	74	64	86	85	85
10	64	88	96	33	42	60	96	22	63	34	87	77	95	94	94
15	69	95	100	35	45	66	99	25	65	37	91	83	99	99	99
20	69	98	101	36	48	70	100	26	67	39	95	85	101	102	102
25	70	98	103	36	49	71	100	28	68	39	96	86	102	103	103
30	70	99	103	35	48	71	101	28	68	40	96	88	103	103	103
35	70	99	103	36	48	72	101	28	69	39	98	88	105	105	105
40	70	99	103	35	48	72	101	28	68	40	98	88	104	105	105
45	70	99	103	35	48	72	101	28	68	40	98	88	105	105	105
50	70	99	103	35	48	72	101	28	68	40	98	88	105	105	105
55	70	99	103	35	48	72	101	28	68	40	98	88	105	105	105
60	70	99	103	35	48	72	101	28	68	40	98	88	105	105	105
Temperature, °C.....	25.1	25.1	25.1	25.0	25.0	25.1	25.0	25.1	25.1	25.1	25.1	24.9	25.1	24.9	25.1
Total volume O ₂ absorbed, cc.....	0.230	0.331	0.338	0.115	0.157	0.236	0.331	0.092	0.223	0.131	0.321	0.289	0.321	0.344	0.344
Total oxidation when absorption ceased, per cent.	64.8	73.1	93.5	32.4	44.4	66.6	93.5	25.9	62.9	37.0	90.7	81.5	97.2	97.2	97.2

TABLE II

Effect of Small Amounts of Iron

The figures are for absorption, measured as mm. difference of manometer level.

pH.....	7.4	7.4	7.4	7.4	7.6	7.6	7.6	7.6	8.0	8.0	8.0	8.0
Time shaken	Dialuric acid alone	+ 0.0001 mg. Fe	+ 0.001 mg. Fe	+ 0.005 mg. Fe	Dialuric acid alone	+ 0.0001 mg. Fe	+ 0.001 mg. Fe	+ 0.005 mg. Fe	Dialuric acid alone	+ 0.0001 mg. Fe	+ 0.001 mg. Fe	+ 0.005 mg. Fe
<i>min.</i>												
0	0	0	0	0	0	0	0	0	0	0	0	0
5	68	76	86	86	16	27	50	85	40	54	71	83
10	81	87	96	97	21	34	58	95	48	62	81	94
15	86	94	100	101	22	37	62	99	52	65	87	97
20	87	97	102	103	23	38	63	104	53	66	89	101
25	89	97	103	104	24	39	65	104	55	67	89	101
30	90	99	104	104	24	40	64	105	54	67	90	102
35	90	100	106	106	24	40	65	105	55	68	90	104
40	90	100	105	106	24	40	65	105	55	68	91	104
45	90	100	105	106	24	40	65	105	55	68	91	104
50	90	100	105	106	24	40	65	105	55	68	91	104
55	90	100	105	106	24	40	65	105	55	68	91	104
60	90	100	105	106	24	40	65	105	55	68	91	104
Temperature, °C.....	25.1	25.0	25.0	25.0	25.1	24.9	24.9	25.0	25.1	25.0	25.0	25.0
Total volume O ₂ absorbed, cc.....	0.295	0.328	0.344	0.348	0.079	0.131	0.213	0.344	0.180	0.223	0.298	0.341
Total oxidation when absorption ceased, per cent.....	83.2	92.6	97.2	98.1	22.2	37.0	60.1	97.2	50.9	62.9	84.2	96.2

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10 mg. samples of iron-free dialuric acid was measured, and a similar series in which 0.0001 mg., 0.001 mg., and 0.005 mg. of iron, respectively, were added to equal amounts of dialuric acid and the oxidation measured. These oxidations were measured at hydrogen ion concentrations varying from pH 6.0 to 8.0. The iron was added as FeCl_3 , the various concentrations being prepared from a solution containing 0.1 gm. of standard iron wire dissolved in concentrated hydrochloric acid and diluted to a liter.

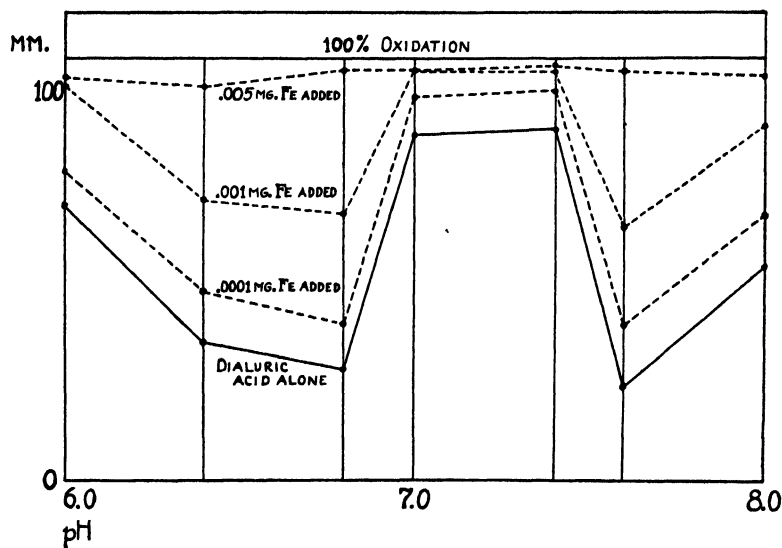


FIG. 1. Comparison of oxidation of dialuric acid alone with dialuric acid in the presence of varying amounts of iron. The abscissas show pH values, the ordinates negative pressure readings after 60 minutes absorption.

The respirometer was shaken throughout an experiment by a shaking machine, moving at a speed of 170 to 180 oscillations per minute, and the temperature in the oxidizing chambers was maintained constant in a water bath at $25.0 \pm 0.1^\circ$.

The results obtained are tabulated in Tables I and II.

DISCUSSION

Fig. 1 shows comparatively the amounts of oxidation of samples of iron-free dialuric acid and of samples to which varying amounts

of iron have been added. The concentration of hydrogen ions is plotted on the abscissa, the variation being confined to the limits of pH 6.0 to pH 8.0. The ordinates represent the mm. of negative pressure due to the absorption of oxygen after 60 minutes of shaking.

Tables I and II and Fig. 1 will show how great is the acceleration by small quantities of iron. It was found that the rates of oxidation of samples of iron-free dialuric acid were somewhat less than those recorded during the preceding work (5), attesting probably the more complete removal of iron, which was present in some of the buffers then used and was responsible for a part of the phenomenal oxidation in certain cases. The addition of 0.0001 mg. of iron produced a decided increase in oxidation rate at pH values other than 7.0 to 7.4; 0.001 mg. of iron, a much greater increase; while the addition of 0.005 mg. of iron was sufficient to produce almost complete oxidation, even at unfavorable pH values. In fact, the oxidations as accelerated by the addition of 0.005 mg. of iron may be considered to be complete, a slight divergence from the theoretical possible absorption being due possibly to some limiting factor in manipulation. The apparatus was not shaken at a maximum speed, and it is possible that oxygen could not enter the solution fast enough. At least, it can be seen in the oxidations at pH 7.0 and 7.4 that maximum absorption is obtained, for here, the acceleration due to 0.005 mg. of Fe is very small and coincides with that produced by 0.001 mg. of Fe.

Iron, in this oxidation, undoubtedly acts the part of an oxygen carrier, as it is supposed to do in the cell. The mechanism of this acceleration possibly involves the formation of an intermediate compound of ferric iron and dialuric acid. Ferric iron may unite with the dialuric acid to oxidize it to alloxan, the compound immediately breaking up and the iron becoming free again as ferrous iron. The oxygen of the air oxidizes the iron back to the ferric state and the process is repeated.

II. Action of Cyanides

Closely related to the subject of acceleration by iron is the one of inhibition by cyanides. It has been shown by many investigators that small concentrations of KCN have the power to depress cellular oxidations. Mathews and Walker (11) showed that very

small amounts of KCN were sufficient to check or prevent the spontaneous oxidation of cysteine to cystine. More recently, Gerwe (4) has shown that HCN inhibits only the accelerating action of iron, and not the spontaneous oxidation of cysteine. He thus confirmed the theory especially advocated by Warburg that cyanides inhibit respiration by combining with the iron. Warburg has maintained the view that if the oxidative process of any system is paralyzed by cyanide, then iron may be assumed to be the chief catalytic agent involved.

Many cases, however, have been reported where cyanide had no effect at all on oxidation. Thunberg (12) states that succino-dehydrogenase in muscle was not inhibited by cyanide in its methylene blue-reducing power in the presence of succinic acid, whereas strong inhibition in oxygen uptake was observed. Hopkins and his coworkers also state that the Schardinger enzyme from milk and xanthine oxidase are not at all sensitive toward cyanide. They concluded, therefore, that those oxidation systems had nothing to do with iron. Szent-Györgyi (13) has recently shown that the hexoxidase from cabbage leaves, probably related to the highly oxidizable hexuronic acid in the suprarenal cortex of animals, is not sensitive to cyanide.

Gerwe (4) has found that cyanides inhibit the acceleration of the cysteine oxidation by added iron, but that cysteine, entirely free from iron, is not affected in its rate of oxidation and is truly autoxidizable. This disproves Warburg's early supposition that cysteine had no true autoxidation. Cyanides stop the oxidation of cytochrome from yeast, described by Keilin (14) as an intracellular respiratory catalyst, but here again we are dealing with a compound containing iron. Cytochrome has as a nucleus an iron-pyrrole compound, similar to that in hemoglobin. Elvehjem (15) finds that the respiration of yeast is 80 per cent inhibited by KCN, but this is not startling as it has already been shown that iron, and especially cytochrome, is an essential element in the growth and metabolism of yeast.

Consequently, it is of importance to discover whether the oxidation of dialuric acid is affected by cyanides. In this way its autoxidizability can be confirmed, because if this oxidation is a catalysis by iron, it should be inhibited by cyanides.

Method and Results

A series of experiments was carried out in which varying concentrations of KCN were added to oxidizing mixtures containing 10 mg. samples of dialuric acid free from iron. The concentrations used were 0.00005 M, 0.0001 M, 0.0002 M, 0.0005 M, 0.001 M, 0.002 M, 0.005 M, and 0.02 M. These were the final concentrations in the solutions and the oxidations were measured at pH 7.0 and

TABLE III
Effect of Cyanide on Oxidation of Dialuric Acid

pH	Temperature	O ₂ uptake of 10 mg. dialuric acid in mm. after 60 min.	KCN added to make final concentration	Uptake after addition of cyanide
	°C.	c.mm.	M	c.mm.
7.0	25.0	88.0	0.0001	89.0
7.0	25.0	89.0	0.0002	88.5
7.0	25.1	88.0	0.00005	88.5
7.0	25.0	88.5	0.0005	89.0
7.4	25.1	90.5	0.001	90.0
7.4	24.8	90.0	0.002	90.5
7.4	25.0	90.0	0.005	89.5
7.4	25.0	89.5	0.02	90.0

TABLE IV
Effect of Cyanides on Iron Catalysis

pH	O ₂ uptake of 10 mg. of dialuric acid	Fe added as FeCl ₃	KCN added to make solution	O ₂ uptake
	c.mm.	mg.	M	c.mm.
7.0	88.0	0.0001	0.0005	89.0
7.0	88.5	0.001	0.0005	89.0
7.0	88.0	0.005	0.0005	88.5

7.4. In no case was there any inhibition of oxygen uptake (see Table III) and these results show definitely that cyanides do not inhibit the oxidation of pure dialuric acid. These results support those of Richardson and Cannan (16), who found that the addition of HCN had no notable effect on the oxidation.

In order to determine the effect of cyanide on the oxidation of dialuric acid to which iron had been added, 0.005 M KCN was

added to reaction mixtures containing, in addition to the 10 mg. of dialuric acid, 0.0001 mg., 0.001 mg., and 0.005 mg. of iron, respectively, and the oxygen uptake measured. These mixtures, then, contained KCN in the final concentration of 0.0005 M and were buffered at pH 7.0. The oxygen uptake in these instances was just the same as when pure dialuric acid was used alone without KCN (see Table IV).

Altogether, then, pure iron-free dialuric acid oxidizes at the same rate in the presence of varying amounts of KCN as in its absence. Cyanide inhibits the oxidation in the presence of iron only to the extent that the reaction has been catalyzed by iron.

SUMMARY

1. The spontaneous oxidation of dialuric acid, occurring at unfavorable pH values, is greatly accelerated by the addition of small amounts of iron; but little or no acceleration by iron was obtained at pH 7.0 to 7.4. This may have been due to the fact that oxygen could not enter the solution fast enough at pH 7.0 to 7.4 to permit any acceleration.

2. The autoxidation of iron-free dialuric acid is not inhibited by cyanides.

3. The addition of KCN to dialuric acid of which the oxidation was catalyzed by iron reduced the oxygen consumption to that of dialuric acid alone.

4. In its acceleration by iron and the inhibition of iron catalysis by cyanide, the spontaneous oxidation of dialuric acid shows the same interesting parallelisms to the oxidations of cells as are shown by cysteine and its compounds, such as glutathione.

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AN IMPROVED LACTIC ACID APPARATUS*

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An improved form of lactic acid apparatus for use in the permanganate and manganese dioxide oxidation methods (1) has been used in these laboratories the past 2 years. Its advantages are (1) the incorporation of a more efficient type of condensing unit which assures adequate cooling within a wide range of tap water temperature and makes the apparatus more compact and sturdy; and (2) the use of rubber connections is minimized.

To make these advantages available to others, the short description and figure are published.

The apparatus is illustrated in Fig. 1, and consists of the three units, boiling flask, condenser, and absorber, all of Pyrex glass. The boiling flask *C*, a wide mouth extraction flask of 250 cc. capacity, is fitted by a rubber stopper to a condenser unit made from a West type condenser (2). The condenser should have an inner tube of thin wall Pyrex, about 7 mm. inside diameter with 0.5 to 1 mm. space between the inner and outer tubes (water space) and a water column around 425 mm. long. A standard wall Pyrex tube (about 8 mm. outside diameter) is sealed to the condenser and bent for attachment to the absorber *B*, as shown in Fig. 1. The absorber consists of a 150 cc. wide mouth extraction flask fitted with a tube *G*, containing a few beads as shown. *G* is conveniently made from a 25 × 200 mm. Pyrex test-tube, into the bottom of which a 7 × 140 mm. tube is sealed. *D*, the permanganate or manganese dioxide container, is prepared from a small

* Demonstrated at The Thirteenth International Physiological Congress, August 19-23, 1929, Boston.

The complete apparatus may be obtained from Arthur H. Thomas Company, Philadelphia.

Pyrex test-tube and attached by rubber tubing to the delivery tube, *H*. *F* is a screw clamp to control the flow of oxidizing agent into the flask. A small hole, cut in the rubber below *F*, serves as

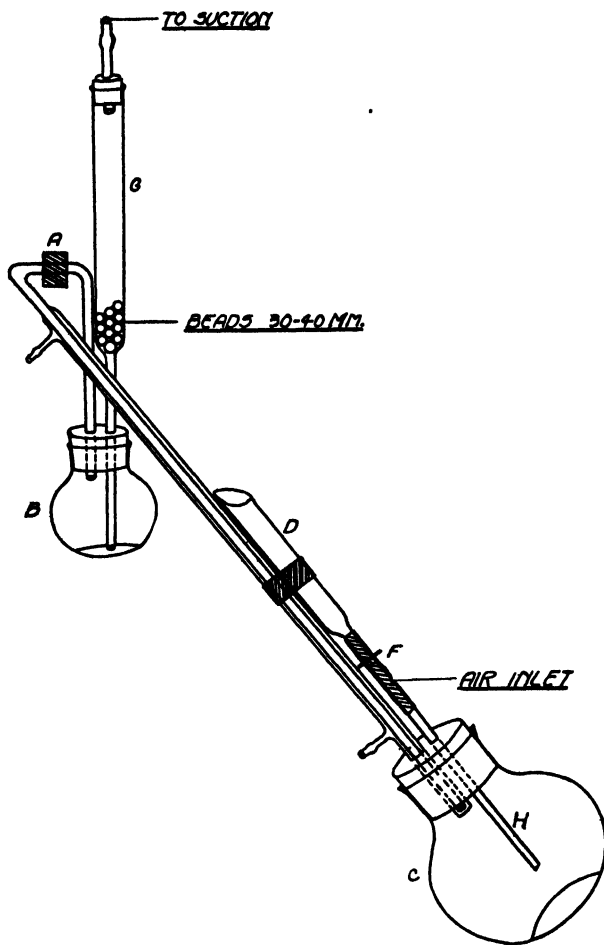


FIG. 1

an air inlet. *D* is fastened to the condenser with friction tape. The apparatus is loosely supported in the split cork, *A*, by a clamp or by friction tape to a horizontal rod, and under the neck of the

boiling flask or low on the condenser by a horizontal rod. The slope of the condenser, relative to the desk top, should be about 45° . Mounting in this way insures freedom of movement in the vertical plane without strains. A micro burner is used to heat the boiling flask.

Several units may be compactly assembled in a row on parallel horizontal rods, one attached loosely at *A*, and the other simply passing under the lower part of the condensers.

The condenser units may be more simply made by fastening the outer jacket in place with rubber connections. The inner tube is extended beyond the jacket and bent to enter the absorber as explained above. Such condensers, while not quite so efficient as the all glass ones with the thin inner tubes, are entirely satisfactory.

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THE PARTIAL DEHYDROGENATION OF URSOLIC ACID

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In our studies on the partial dehydrogenation of β -amyrine¹ and hederagenin² and their derivatives it has been found that these substances show a close analogy in their behavior on heating with sulfur. Partial dehydrogenation may be accomplished in each case with the formation of cyclic sulfur compounds. These sulfur compounds exhibited similar properties and on oxidation gave rise to analogous sulfur-free substances which warrant the conclusion that analogous if not identical structures are present in both β -amyrine and hederagenin which are the points attacked by the sulfur. In the case of α -amyrine,¹ however, apparently only 1 mol of hydrogen was removed with the formation of a sulfur-free dehydro- α -amyrine which was characterized by its high positive rotation ($[\alpha]_D = +358^\circ$). The divergent behavior of α -amyrine from the former substances indicated a definite structural difference.

In continuing our studies on the partial dehydrogenation of polyterpenes by an extension to other members of the group, another substance, ursolic acid, has been found to belong in the same category as α -amyrine, as regards its behavior towards sulfur. The present work was practically completed when the recent article of Sando³ appeared. The latter confirmed the previous conclusion of van der Haar⁴ that the three substances, malol, prunol, and ursolic acid of different origin are identical. Contrary, however, to van der Haar's formulation of $C_{31}H_{50}O_3$, Sando has concluded from numerous analyses that $C_{30}H_{48}O_3$ appears to

¹ Jacobs, W. A., and Fleck, E. E., *J. Biol. Chem.*, **88**, 137 (1930).

² Jacobs, W. A., and Fleck, E. E., *J. Biol. Chem.*, **88**, 153 (1930).

³ Sando, C. E., *J. Biol. Chem.*, **90**, 477 (1931).

⁴ van der Haar, A. W., *Rec. trav. chim. Pays-Bas*, **43**, 542, 548 (1924).

be the correct formula. In reporting our own analytical results we are giving this conclusion merely provisional acceptance.

In our first experiments the attempt was made to employ ursolic acid and its methyl ester directly for partial dehydrogenation with sulfur. Extensive carbonization, however, was found to occur and following our experience in other cases we turned to derivatives of the ester in which the apparently disturbing hydroxyl was removed. In one case *ursolic methyl ester benzoate* was employed. On being heated with sulfur a substance was formed apparently with the loss of 1 mol of hydrogen. The resulting *dehydroursolic methyl ester benzoate* was characterized by a high positive rotation ($[\alpha]_D = +241^\circ$).

In another series of experiments the hydroxyl group of ursolic acid was shown to be of secondary character since the ester on oxidation in acetic acid solution with chromic acid yielded a ketone, *ursonic methyl ester*, which was characterized by its *oxime*. If the oxidation mixture contained sulfuric acid (Kiliani CrO_3 solution), the oxidation proceeded further with the formation apparently of a *diketone*, $\text{C}_{30}\text{H}_{46}\text{O}_4$. From the latter, however, only a *monooxime* could be obtained. The keto group of ursonic methyl ester was readily reduced by the Clemmensen method to CH_2 with the formation of *ursanic methyl ester* in which the ester group was still very resistant to saponification. Only by vigorous treatment with alkali could it be saponified to *ursanic acid*.

When ursanic methyl ester was heated with sulfur at $230\text{--}240^\circ$ partial dehydrogenation occurred and from the reaction mixture a substance was obtained which analysis indicated to possess the formula $\text{C}_{30}\text{H}_{46}\text{O}_2$ and which had been formed apparently by the loss of 1 mol of hydrogen. This substance, *dehydroursanic methyl ester*, like dehydroursolic methyl ester benzoate and α -amyrine, possessed a strong positive rotation, $[\alpha]_D = +288^\circ$. Like its parent ester, it was saponified with difficulty to *dehydroursanic acid*.

It appears, therefore, that triterpene derivatives, as regards their behavior on partial dehydrogenation with sulfur, may be separated at least into two categories. The difference in structure responsible for this divergent behavior of the two groups is now under investigation.

EXPERIMENTAL

Ursolic Acid—Crude ursolic acid which melted at 279–280° (corrected) was isolated in the form of colorless needles from *Arctostaphylos uva-ursi* leaves essentially by the method of Dodge.⁵

For molecular weight determinations and analysis the ursolic acid was purified, through the sodium salt, by the method of Sando.³ The final product melted at 284–285° (corrected).⁶

4.040 mg. substance: 3.875 mg. H₂O, 11.658 mg. CO₂.
 3.925 “ “ : 3.800 “ “ 11.350 “ “
 C₃₀H₄₈O₃. Calculated. C 78.88, H 10.61
 Found. (a) “ 78.70, “ 10.73
 (b) “ 78.86, “ 10.83

Ursolic Methyl Ester—When crude ursolic acid was esterified in ether solution with diazomethane complete solution occurred at once. After removal of the solvent the residue was fractionated from acetone. Two substances which differed in solubility were separated by this procedure. The less soluble substance formed needles which melted at 221–222°.

$[\alpha]_D^{25} = +59^\circ$ (c = 1.027 in pyridine).
 5.075 mg. substance: 4.965 mg. H₂O, 14.947 mg. CO₂.
 5.225 “ “ : 5.130 “ “ 15.390 “ “
 5.565 “ “ : 1.395 “ AgI.
 Found. (a) C 80.31, H 10.95
 (b) “ 80.33, “ 10.98
 (c) OCH₃ 3.31

Molecular weight determination was made according to the method of Rast. 28.130 mg. of camphor: 2.663 mg. of anhydrous substance, $\Delta = 9.4^\circ$. Molecular weight found, 453.

This substance was recovered unchanged after boiling with 10 per cent alcoholic KOH for 4 hours. The above methoxyl figure is about one-half of that for ursolic methyl ester itself. This observation, together with the fact that the substance yields on oxidation with CrO₃ about one-half of the amount of ursonic methyl ester given by pure ursolic methyl ester, indicates that the

⁵ Dodge, F., *J. Am. Chem. Soc.*, **40**, 1932 (1918).

⁶ When the uncorrected melting point of this substance was taken in the usual manner it was found to be 275–276°. The above value was found after correcting for projecting thermometer stem.

substance is possibly a molecular association compound of ursolic methyl ester and some other substance of the same molecular order. The substance has not been investigated further.

The more soluble substance yielded a substance which melted at 110–120° and corresponded in all respects with the monohydrate of ursolic methyl ester as previously described.^{3,7} The yield was 5.5 gm. from 10 gm. of crude ursolic acid.

$[\alpha]_D^{25} = +58^\circ$ ($c = 1.030$ in pyridine).

4.345 mg. substance: 4.190 mg. H₂O, 12.615 mg. CO₂.

4.270 " " : 4.025 " " 12.380 " "

5.250 " " : 2.500 " AgI.

C₃₁H₅₀O₃. Calculated. C 79.08, H 10.71, OCH₃ 6.59

Found. (a) " 79.19, " 10.79

(b) " 79.07, " 10.55

(c) OCH₃ 6.30

Benzoate of Ursolic Methyl Ester—Ursolic methyl ester was benzoylated in pyridine solution with benzoyl chloride. The benzoate formed needles from acetone which melted at 212–213°. After the melt was allowed to resolidify it melted at 235–236°.

By the Liebermann-Burchard procedure it gave a pink color which changed to a greenish brown when the solution was warmed.

$[\alpha]_D^{25} = +70^\circ$ ($c = 1.035$ in pyridine).

4.310 mg. substance: 3.630 mg. H₂O, 12.520 mg. CO₂.

4.730 " " : 3.995 " " 13.790 " "

6.072 " " : 2.435 " AgI.

C₃₃H₅₄O₄. Calculated. C 79.38, H 9.47, OCH₃ 5.39

Found. (a) " 79.22, " 9.42

(b) " 79.52, " 9.46

(c) OCH₃ 5.30

Dehydroursolic Methyl Ester Benzoate—A mixture of 0.5 gm. of ursolic methyl ester benzoate and an equal amount of sulfur was heated at 230–240° for 3½ hours in an atmosphere of nitrogen. The reaction mass was extracted with ether and the extract was evaporated to dryness. The residue was dissolved in a small amount of ether and the sulfur which remained was filtered off. After concentrating the filtrate this process was repeated until complete solution of the residue occurred. The final residue was

⁷ Power, F. B., and Moore, C. W., *J. Chem. Soc.*, **97**, 1106 (1910). van der Haar, A. W., *Rec. trav. chim. Pays-Bas*, **43**, 375 (1924).

then dissolved in alcohol and the solution was decolorized with Darco. For final purification it was recrystallized from acetone from which it crystallized as needles which melted at 210–212°.

The cholesterol test showed a deep purple color which changed to a dark brown when the solution was warmed.

$[\alpha]_D^{25} = +241^\circ$ ($c = 1.010$ in pyridine).

4.963 mg. substance: 3.990 mg. H_2O , 14.525 mg. CO_2 .

4.968 " " : 4.102 " " 14.527 " "

$C_{33}H_{52}O_4$. Calculated. C 79.66, H 9.15

Found. (a) " 79.82, " 9.01

(b) " 79.75, " 9.24

Ursonic Methyl Ester—A solution of 5 gm. of ursolic methyl ester in 50 cc. of acetic acid was added to an acetic acid solution of 1 gm. of CrO_3 , and the mixture was heated on the steam bath for $\frac{1}{2}$ hour. The product was precipitated with 3 volumes of water. For purification the precipitate was recrystallized from acetone from which it separated in the form of prisms which melted at 192–193°. Ursonic methyl ester is more soluble in ether and ligroin (80–90°) than in petroleic ether, alcohol, and acetone.

The cholesterol test gave a pink color which on warming changed to a light blue becoming finally a light green.

$[\alpha]_D^{25} = +84^\circ$ ($c = 1.010$ in pyridine).

4.640 mg. substance: 4.285 mg. H_2O , 13.535 mg. CO_2 .

4.352 " " : 4.002 " " 12.700 " "

5.175 " " : 2.685 " AgI.

$C_{31}H_{48}O_3$. Calculated. C 79.42, H 10.33, OCH_3 6.61

Found. (a) " 79.56, " 10.33

(b) " 79.59, " 10.29

(c) OCH_3 , 6.85

Ursonic Methyl Ester Oxime—The oxime was prepared in the usual manner in alcoholic solution. It formed needles from methyl alcohol which melted at 243–244°.

3.850 mg. substance: 3.620 mg. H_2O , 10.920 mg. CO_2 .

4.080 " " : 2.030 " AgI.

6.017 " " : 0.166 cc. N_2 (30° and 752.7 mm.).

$C_{31}H_{49}O_2N$. Calculated. C 76.96, H 10.19, OCH_3 6.41, N 2.90

Found. (a) " 77.35, " 10.52

(b) OCH_3 , 6.56

(c) N 3.08

The Diketone, $C_{31}H_{46}O_4$ —A solution of 0.5 gm. of ursolic methyl ester in 0.5 cc. of chloroform and 100 cc. of acetic acid was treated with 25 cc. (a large excess) of Kiliani's chromic acid solution. The temperature of the reaction was not allowed to rise above 28° . At the end of $\frac{1}{2}$ hour the solution was diluted with 3 volumes of water and then extracted with ether. The extract was washed with dilute alkali. The alkaline extract when acidified gave a small amount of non-crystallizable gum.

The ether solution of the neutral portion gave a residue which was crystallized from dilute alcohol. It separated as stout prisms which melted at 176 – 177° .

The cholesterol test gave an orange-yellow color which deepened on warming.

$[\alpha]_D^{25} = +128^\circ$ ($c = 1.017$ in pyridine).

3.486 mg. substance: 2.995 mg. H_2O , 9.855 mg. CO_2 .

3.615 " " : 3.040 " " 10.210 " "

6.225 " " : 3.060 " AgI.

$C_{31}H_{46}O_4$. Calculated. C 77.12, H 9.60, OCH_3 6.42

Found. (a) " 77.10, " 9.61

(b) " 77.01, " 9.41

(c) OCH_3 6.48

Monooxime of Diketone, $C_{31}H_{46}O_4$ —This was prepared in the usual manner in alcoholic solution. The oxime was recrystallized from methyl alcohol from which it separated as flat prisms which melted at 210 – 211° .

3.877 mg. substance: 3.380 mg. H_2O , 10.680 mg. CO_2 .

4.365 " " : 2.100 " AgI.

6.405 " " : 0.169 cc. N_2 (29° and 753.5 mm.).

$C_{31}H_{47}O_4N$. Calculated. C 74.80, H 9.53, OCH_3 6.23, N 2.81

Found. (a) " 75.13, " 9.75

(b) OCH_3 6.40

(c) N 2.96

Ursanic Methyl Ester—A solution of 2 gm. of ursonic methyl ester in 320 cc. of acetic acid and 80 cc. of HCl (1.19) was added to 50 gm. of amalgamated zinc. The mixture was refluxed gently for 1 hour. The reaction mass was diluted with 3 volumes of water and the resulting precipitate was collected with water. For analysis this product was recrystallized from methyl alcohol. It separated as needles which melted at 117 – 118° . Ursanic

methyl ester is very soluble in ether, acetone, and ligroin. It is less soluble in alcohol and least soluble in methyl alcohol.

The cholesterol test gave a pink color which changed to light brown on warming.

$[\alpha]_D^{25} = +65^\circ$ ($c = 1.020$ in pyridine).

4.218 mg. substance: 4.260 mg. H_2O , 12.683 mg. CO_2 .

3.777 " " : 3.800 " " 11.320 " "

4.393 " " : 2.145 " AgI.

$C_{31}H_{48}O_2$. Calculated. C 81.87, H 11.09, OCH_3 6.82

Found. (a) " 82.01, " 11.30

(b) " 81.74, " 11.25

(c) OCH_3 6.45

Ursanic Acid—A solution of 0.1 gm. of ursanic methyl ester in 10 cc. of 20 per cent alcoholic KOH was heated in a sealed tube at 120 – 130° for 3 hours. The precipitate of salt and unchanged ester, which separated when the reaction mixture was diluted with 3 volumes of water, was filtered and dried. This dried material was extracted several times with dry ether in order to remove unchanged ester. The insoluble potassium salt was suspended in ether and brought into solution by the addition of a few drops of HCl. The washed ether solution was evaporated and the residue was recrystallized from dilute acetone. Ursanic acid separated as needles which melted at 223 – 225° .

3.967 mg. substance: 3.990 mg. H_2O , 11.853 mg. CO_2 .

3.485 " " : 3.378 " " 10.395 " "

$C_{30}H_{48}O_2$. Calculated. C 81.74, H 10.99

Found. (a) " 81.49, " 11.25

(b) " 81.35, " 10.85

Dehydroursanic Methyl Ester—A mixture of 0.5 gm. of ursanic methyl ester and an equal amount of sulfur was heated in an atmosphere of nitrogen at a temperature of 230 – 240° for $2\frac{1}{2}$ hours. At the end of this period H_2S production had practically ceased. The reaction mass was extracted with ether and the residue obtained after removal of the ether was distilled at 2 mm. The distillation was interrupted when the temperature of the metal bath had reached 275° . At this point practically all of the sulfur had distilled. When the temperature of the bath was maintained between 275 – 310° a resin distilled. This resin crystallized when

dissolved in methyl alcohol. Repeated recrystallizations from methyl alcohol yielded prisms which melted at 110–111°. The yield was about 20 per cent.

The cholesterol test gave a deep violet color which changed to a dark brown when the solution was warmed.

$[\alpha]_D^{25} = +288^\circ$ ($c = 1.020$ in pyridine).

4.623 mg. substance: 4.420 mg. H_2O , 13.932 mg. CO_2 .

3.943 " " : 3.780 " " 11.890 " "

4.985 " " : 2.450 " AgI.

$C_{31}H_{48}O_2$. Calculated. C 82.23, H 10.70, OCH₃ 6.85

Found. (a) " 82.19, " 10.70

(b) " 82.24, " 10.73

(c) " " " OCH₃ 6.50

Dehydroursanic Acid—A solution of 0.1 gm. of dehydroursanic methyl ester in 10 cc. of 20 per cent alcoholic KOH was heated in a sealed tube at 120–130° for 3 hours. The reaction mixture was treated in the same manner as given in the case of ursanic acid. Final recrystallization from dilute acetone yielded needles which melted at 174–176°.

3.770 mg. substance: 3.560 mg. H_2O , 11.355 mg. CO_2 .

$C_{30}H_{46}O_2$. Calculated. C 82.13, H 10.57

Found. " 82.15, " 10.57

A SIMPLIFICATION OF THE OKEY METHOD FOR THE DETERMINATION OF CHOLESTEROL BY OXIDATION OF THE DIGITONIDE

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The original method described by Okey (1) for the determination of cholesterol by means of oxidation of the digitonide is an intricate procedure especially in the precipitation of the cholesterol digitonide. A high degree of accuracy can be attained only after considerable training and experience. The need for special and difficultly obtainable apparatus and for practice in the technique of making numerous transfers and filtering and washing small amounts of material discourages general application of the method. A simplification has been developed in which the precipitation, washing, and oxidation are accomplished in the one centrifuge tube.

Special Apparatus

15 cc. centrifuge tubes with conical bottoms and with well fitted ground stoppers were used.¹

Procedure

An aliquot of the alcohol-ether extract prepared according to Bloor's (2) method and containing 0.5 to 1.5 mg. of free cholesterol is placed in the 15 cc. centrifuge tube. Precipitation is most favorable if the volume is 6 to 8 cc., and if the volume of extract taken exceeds this amount, it is reduced by placing it in a water bath at about 70°. 1 cc. of 1 per cent alcoholic digitonin solution is added and the whole is evaporated to dryness in an oven at 124° or in a water bath at 70°. The latter procedure requires much

¹ These are easily prepared by grinding glass stoppers in ordinary centrifuge tubes with the aid of an abrasive.

longer time but is safer. If the oven is small or if the number of tubes is large, the possibility of the formation of an explosive mixture must be borne in mind. This has never happened during the course of more than 200 determinations. If the oven is well ventilated and if the heating units and spark control are well separated from the oven chamber, the danger is slight. Removal of the ether on the water bath and of the alcohol in the oven also lessens the risk of an explosion. If the water bath temperature is above 70°, there are bumping and loss of material. In the electric oven, evaporation is completed in 10 to 15 minutes. When the last traces of solvent have been removed, 10 cc. of redistilled ethyl ether are added at once, without removal of the digitonide from the sides of the tube. If the initial washing is accomplished without disturbing the precipitate, it becomes flaky and allows thorough subsequent washings. Two additional extractions are made with warm ether by mixing the digitonide and ether with a stirring rod. The solvent is removed each time by decantation after centrifugation at high speed to pack the solid material in the bottom of the tube. The last traces of ether are removed by warming the tube in a water bath, and the precipitate is then washed with warm distilled water until the foamy appearance is absent. The water is decanted in the same manner as the ether but the centrifugation must be carried on for a longer time ($\frac{1}{2}$ hour) to insure packing of the precipitate which has a greater tendency to remain in suspension in water than in ether. The residual moisture is then removed by drying in the oven.

The oxidative mixture is added and intimate contact with the precipitate effected by placing a short (2 mm. diameter) stirring rod in the tube and tapping the tube carefully. The stirring rod is not removed during the heating and must be short enough to allow the stoppers to be inserted. The tube is placed in the oven for the customary period after which the contents are washed into an Erlenmeyer flask, diluted, and titrated in the usual manner.

Table I shows the figures for the recovery of free cholesterol from artificial and natural plasma extracts as well as comparison figures with a commonly used colorimetric method. The limits of error in the oxidative method are +2 to +4 per cent for 0.5 to 1.5 mg. of free cholesterol, by the colorimetric method +8 to +12 per cent.

In the estimation of total cholesterol, the petroleum ether extract is transferred to the centrifuge tube and evaporated to dryness at room temperature. The residue is taken up in alcohol and the cholesterol precipitated as the digitonide, as just described.

The method is applicable to the determination of sterols in colored plant extracts, the physical nature of which makes the

TABLE I
Typical Determinations of Cholesterol

Nature of sample	Cholesterol present	No. of determinations	Cholesterol found	Error	Limits of error	Type of procedure
	mg.		mg.	per cent	per cent	
Artificial plasma extract*	0.5	8	0.52	+4.0	+3.0 to + 5.0	Oxidized in oven 124°, 15 min.
	1.0	10	1.022	+2.2	+1.1 " + 3.1	
	1.25	10	1.278	+2.4	+1.6 " + 4.0	
	2.0	8	2.064	+4.3	+2.4 " + 6.0	Oxidized in oven 124°, 15 min. No stoppers Colorimetric method of Autenrieth and Funk (3)
	1.25	6	1.19	-5.15	-2.5 " - 7.0	
	1.25	6	1.35	+8.3	+6.5 " +12.0	
10 cc. plasma extract		3	1.02			Oxidized in oven 124°, 15 min.
10 cc. plasma extract + 0.5 mg. free cholesterol	1.52	8	1.55	+2.9	+2.0 to + 3.8	
10 cc. plasma extract + 0.75 mg. free cholesterol	1.77	8	1.82	+3.2	+2.0 " + 3.8	

* Contained free cholesterol, lecithin, and fatty acids in an alcohol-ether solution.

usual methods difficult if the amount available is small. The presence of pigments prevents the use of the colorimetric methods. It has been found that digitonide precipitated from alcoholic solutions of plant extracts may be washed free from all coloring matter and other interfering substances. The ether washings must be thorough and more numerous than in the case of the

animal extracts. Duplicate determinations show agreement within the limits obtained with the plasma extracts.

SUMMARY

A simplification of the Okey method for the determination of cholesterol by means of oxidation of the digitonide is described. The error of the recovery of the free cholesterol averages +2 to +4 per cent. The method is applicable to the determination of free and combined cholesterol, and also lends itself to use in the determination of sterols of plant extracts for which the colorimetric methods may not be used.

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COMPARATIVE STUDIES OF THE METABOLISM OF THE AMINO ACIDS

IV. PHENYLALANINE AND TYROSINE

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In a previous study from this laboratory (1), it was demonstrated that the changes in the distribution of the nitrogenous constituents of the blood following the administration of amino acids were not uniform, but varied with the nature of the amino acids fed or injected. Subsequently it was shown that these differences could be explained in part by the varying rates of absorption of amino acids from the intestinal tract (2). However, it was considered probable that other factors involving the chemical processes of intermediary metabolism, such as differences in the rate of deamination, also played a rôle (3).

The present investigation is concerned with the extension of the earlier work to the aromatic amino acids, tyrosine and phenylalanine. Since these amino acids contain the benzene nucleus and may thus give rise to phenolic derivatives in their intermediary metabolism, the changes in the phenol content of blood and urine were also studied.

EXPERIMENTAL

Male rabbits were used as the experimental animals. The general plan of the experiments, the diets, and the procedures were the same as those previously outlined (1). The urine samples, collected over short periods of time, were obtained by gentle pressure on the abdominal wall or directly by catheterization. Because of the insolubility of the aromatic amino acids, they were converted to the more soluble sodium salts and administered in

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this form. In the earlier experiments (1), the amino acids were fed in amounts equivalent to 0.182 gm. of amino nitrogen (*e.g.* 1 gm. of glycine) per kilo of body weight. The nitrogen contents of phenylalanine and tyrosine are much lower and the amino acids and their sodium salts less soluble than in the case of glycine or alanine. It was therefore difficult to feed or inject these aromatic amino acids in amounts comparable to those used in the earlier experiments. 1 gm. of tyrosine per kilo of body weight was administered and 0.91 gm. of phenylalanine (the molecular equivalent of 1 gm. of tyrosine). This corresponds to 0.078 gm. of nitrogen per kilo, slightly less than half of the amount of nitrogen of the earlier experiments.

Potassium oxalate was used as an anticoagulant. The bloods were deproteinized by the method of Folin and Wu and the total non-protein, urea, and amino acid nitrogens were determined in the filtrates by the usual methods of Folin. Phenols were determined in a separate sample of blood according to the procedure of Theis and Benedict (4). Phenols of the urine were determined by the method of Folin and Denis (5), urinary amino acid nitrogen and creatinine by the methods of Folin, and urinary urea by the urease method of Van Slyke and Cullen.

l-Tyrosine and *dl*-phenylalanine were administered in these experiments. Subsequently *l*-phenylalanine was used in place of the *dl* form, but as the results did not vary significantly from those obtained with the *dl* form, they are not presented in tabular form. A considerable number of experiments was carried out, of which a few typical ones only are shown in the tables.

A study of the distribution of the non-protein nitrogen of the blood together with figures for free "phenol" is presented in Table I. The figures fail to show any significant rise in amino acid nitrogen of the blood at any period, the values resembling in this respect those obtained after the administration of lysine, and glutamic and aspartic acids (1). That this was not due to a failure of absorption from the gastrointestinal tract or from the site of injection is demonstrated by the rise in the "phenol" content of the blood, a rise which was noted without exception in the bloods collected up to 6 hours after the ingestion of the amino acids. It is evident from this and other considerations to be discussed later that the metabolism of the aromatic acids takes place promptly after their en-

TABLE I

Distribution of Non-Protein Nitrogenous Constituents and Free Phenols of Blood Following Administration of Amino Acids

All results are expressed in mg. per 100 cc. of whole blood.

Experiment No. and substance administered	Time	Non-protein N	Urea N	Amino acid N	Free phenol
	<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
XIV-11. <i>l</i> -Tyrosine, 2.60 gm. orally	0	36.4	17.3	7.3	3.2
	3	34.3	20.7	6.3	4.5
	6	34.3	21.4	6.4	4.1
	12	36.4	23.8	6.4	3.4
	30	36.4	24.0	5.5	2.5
VIII-4. <i>l</i> -Tyrosine, 2.15 gm. orally	0	28.2	9.8	6.4	3.1
	3	39.0	12.8	7.5	8.7
	6	39.0	11.9	6.6	7.5
	12	35.2	10.1	5.7	4.0
	30	31.6	10.3	6.1	3.5
IX-5. <i>dl</i> -Phenylalanine, 2.84 gm. orally	0	29.8	16.1	7.3	3.6
	3	37.0	17.0	9.4	6.0
	6	32.8	17.0	11.6	5.0
	12	41.4	20.7	8.9	4.3
	30	34.8	16.5	8.3	4.2
V-2. <i>dl</i> -Phenylalanine, 2.06 gm. orally	0	37.5	13.6	8.0	2.9
	3	42.5	17.2	12.0	4.0
	6	40.0	18.8	9.9	3.6
	12	41.3	21.1	9.8	3.0
	30	39.8	19.6	7.4	2.8
X-6. <i>dl</i> -Phenylalanine, 3.37 gm. subcutaneously	0	32.6	16.0	8.0	2.0
	3	34.7	21.7	9.2	2.9
	6	32.7		8.0	2.7
	12	32.4	17.7	6.8	2.1
	30	32.6	17.7	5.9	1.6
VI-4. <i>dl</i> -Phenylalanine, 2.06 gm. subcutaneously	0	36.4	15.4	8.8	1.5
	3	39.2	18.2	8.8	2.8
	6	38.7	20.0	7.8	2.0
	12	35.1	20.1	8.1	1.7
	30	31.1	16.0	7.2	1.5

trance into the blood stream. Although the figures for amino acid nitrogen did not show any significant increase, a slight increase in

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TABLE II

Influence of Oral and Subcutaneous Administration of dl-Phenylalanine and Tyrosine on Urinary Excretion

Rabbit; weight, 2.30 kilos.

Day of experiment	Length of period	Urine			Blood*
		Creatinine	Amino N	Total phenol	Free phenol
	hrs.	gm.	gm.	gm.	mg.
1	12	0.057	0.008	0.063	2.2
	12	0.055	0.007	0.061	
Total.....	24	0.112	0.015	0.124	
2†	6	0.037	0.014	0.071	3.9‡
	6	0.028	0.005	0.050	
	12	0.058	0.010	0.063	
Total.....	24	0.123	0.029	0.184	
3	12	0.054	0.008	0.053	
	12	0.052	0.005	0.051	
Total.....	24	0.106	0.013	0.104	
4	12	0.056	0.014	0.050	2.3
	12	0.056	0.010	0.041	
Total....	24	0.112	0.024	0.091	
5§	6	0.027	0.002	0.051	5.9‡
	6	0.028	0.003	0.051	
	12	0.052	0.004	0.042	
Total....	24	0.107	0.009	0.144	
6	12	Specimen lost			
	12				
Total.....	24	0.053	0.004	0.040	
7	12	0.052	0.005	0.032	2.5
	12	0.059	0.006	0.051	
Total.....	24	0.111	0.011	0.083	
8	6	0.038	0.019	0.051	4.3‡
	6	0.033	0.016	0.044	
	12	0.066	0.015	0.066	
Total.....	24	0.137	0.050	0.161	

TABLE II—*Concluded*

Day of experiment	Length of period	Urine			Blood*
		Creatinine	Amino N	Total phenol	Free phenol
	<i>hrs.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>
9	12	0.066	0.010	0.059	
	12	0.060	0.006	0.058	
Total.	24	0.126	0.016	0.117	
10	12	0.055	0.011	0.055	
	12	0.057	0.006	0.048	
Total. ...	24	0.112	0.017	0.103	
11	12	0.050	0.011	0.052	
	12	0.050	0.007	0.052	2.6
Total.	24	0.100	0.018	0.104	

* The results are calculated in mg. per 100 cc.

† At the beginning of this period 2.098 gm. of phenylalanine were administered by tube as the sodium salt.

‡ Blood was taken 3 hours after administration.

§ At the beginning of this period 2.300 gm. of tyrosine were administered by tube as the sodium salt.

|| At the beginning of this period 2.050 gm. of phenylalanine were injected subcutaneously as the sodium salt.

urea nitrogen resulted, an increase which reached its maximum in 12 hours. This increase was not as great as that observed with the other amino acids previously studied (1), but as already pointed out, the amount of nitrogen administered in the present series was less than one-half that of the former series. On the whole, the changes in the partition of the non-protein nitrogen of the blood following the administration of the aromatic amino acids resemble those observed after the administration of the dicarboxylic amino acids and lysine, rather than those observed after the administration of glycine and alanine.

In Table II is presented a protocol, typical of a considerable number, in which the changes in the creatinine, amino acid nitrogen, and total phenols of the urine following the administration of tyrosine and phenylalanine are detailed. It will be noted that except in the experiment in which phenylalanine was injected

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(8th day), there was no appreciable excretion of the amino acid as such by the kidneys, and that after the subcutaneous injection, the excretion although clearly increased above the normal level was not great.

The phenol excretion during the 12 hour period immediately following administration of the phenylalanine was about twice as great as on the control days. By the end of the first 12 hour period this increase in phenol excretion had ceased, so that the excretion for the second 12 hour period on the experimental day was in all cases practically at the normal level. Coincident with the increased phenol elimination in the first 12 hour period of the experimental day there was observed a very slight increase in the excretion of creatinine, as measured by the usual color reaction with picric acid and sodium hydroxide. This increase, so slight as almost to escape detection, was noted particularly in the first 6 hours after the ingestion of the amino acid and seldom amounted to more than 15 mg. (calculated as creatinine) in the 12 hour period. At the same time, an increase in the chromogenic value of the blood in the phenol method of Theis and Benedict was noted in samples of blood collected 3 hours after the beginning of the experimental day.

It was also observed that these same urines, which showed increased chromogenic values in the creatinine method and in the method for phenols, always gave a beautiful blue-green color with dilute solutions of ferric chloride, and that when this color reaction was negative (usually after 12 hours), the increased chromogenic values of the urine in the methods mentioned were no longer to be observed. This suggested that the same substance might be responsible for the ferric chloride test and the apparent increased content of phenol and creatinine. The urine was acidified, extracted with ether, and the ether removed by evaporation at room temperature. The light brown oily residue gave an orange-red color with picric acid and sodium hydroxide, reduced the phosphomolybdic reagent of Folin and Denis in the presence of sodium carbonate, and gave the characteristic green color with ferric chloride. It was possible by repeated extractions with ether to remove completely the material which gave the ferric chloride reaction. Samples of urine from the experimental days, which had shown high values for phenols and creatinine as previously dis-

cussed, after extraction with ether, gave values similar to those of the control periods. It was evident that the apparent increase in creatinine was not due to a greater elimination of this catabolite but to the presence of some other substance reacting similarly in the picric acid test.

The urinary picture after the oral ingestion of tyrosine was different from that discussed above. No green coloration with ferric chloride was ever noted, there was no increase in the chromogenic value of the urine in the creatinine method, but an increase in phenols of both blood and urine was observed. An increase in the phenol content of the urine equivalent to about 5 per cent of the amount of tyrosine ingested was usually noted. Dubin (6) who fed tyrosine to dogs in amounts slightly less in proportion to the body weights than the amounts used in our experiments, noted an increased phenol content of the urine which corresponded to about 15 per cent of the phenol nucleus in the tyrosine administered, as determined by the same method used by us, the Folin-Denis method. The absence of the substance reacting with ferric chloride indicates that the products of metabolism eliminated after tyrosine administration differed from those eliminated after the ingestion of phenylalanine.

Since the green color with ferric chloride is especially characteristic of pyrocatechol and its derivatives, it was suggested in a preliminary report (7) that a diphenol might be an intermediary product in the metabolism of phenylalanine. Further study showed however that phenylpyruvic acid, which might be expected to result from the oxidative deamination of phenylalanine, reduced both the Folin-Denis reagent and the diazotized *p*-nitroaniline used in the phenol method of Theis and Benedict, gave an orange-red color with picric acid and alkali and a green color with ferric chloride. Phenylpyruvic acid was prepared according to the directions of Hemmerle (8) and fed to rabbits in amounts comparable¹ to the amount of phenylalanine used in these experiments. A typical experiment is shown in Table III. The results were similar in every way to those experiments just discussed. It was evident

¹ Because of the instability of the free acid (8), the sodium salt which is stable was prepared and used in the experiments. This salt contains 1 molecule of water of crystallization. Hence, 2.5 gm. of the salt are the molecular equivalent of 2.0 gm. of phenylalanine.

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that the oxidation of the benzene ring in phenylalanine and in phenylpyruvic acid occurred less readily and less completely than we had been led to believe from a study of the literature (9).

Since phenylpyruvic acid reacted with the phosphomolybdic reagent of Folin and Denis, it was hoped that this reaction might be

TABLE III

Influence of Oral Administration of Sodium Phenylpyruvate, Phenylalanine, and Tyrosine on Urinary Excretion

Rabbit; weight, 2.19 kilos.

Day of experiment	Length of period	Urine	
		Creatinine	Total phenols
	<i>hrs.</i>	<i>gm.</i>	<i>gm.</i>
1	24	0.104	0.080
2	24	0.114	0.079
3	24	0.113	0.085
4*	6	0.034	0.058
	18	0.088	0.064
	24	0.122	0.122
5	24	0.114	0.082
6	24	0.120	0.073
7†	6	0.042	0.059
	18	0.076	0.072
	24	0.118	0.131
8	24	0.106	0.073
9	24	0.106	0.076
10‡	6		0.050
	18	0.065	0.082
	24		0.132
11	24	0.117	0.079
12	24	0.106	0.069
13	24	0.107	0.078

* 2.48 gm. of sodium phenylpyruvate were administered orally.

† 2.00 gm. of phenylalanine were administered orally.

‡ 2.19 gm. of tyrosine were administered orally.

used as a quantitative measure of its excretion. It was found, however, that although solutions of phenylpyruvic acid readily reduced the reagent, the color produced was less than that produced by an equivalent amount of phenol ($\frac{1}{15}$ to $\frac{1}{20}$ in some experiments). Moreover, when phenylpyruvic acid was added to

urine, the increase in color in the Folin-Denis phenol method was variable and differed from that which was to be anticipated from the chromogenic value of an aqueous solution of phenylpyruvic acid with the phenol reagent. It appeared that the preliminary procedure in the Folin-Denis method (precipitation of uric acid and other interfering substances by silver lactate in the presence of lactic acid and removal of the excess silver by sodium chloride) influenced in some way the reaction of phenylpyruvic acid and a constant color production could not be obtained. Inasmuch as subsequently another and more satisfactory procedure for the estimation of phenylpyruvic acid (10) was developed, no further attempt to make use of this reaction for the determination of phenylpyruvic acid was made.

Since the apparent rise in the phenol content of the urine after the administration of phenylalanine was not considered to be due to phenols but to the presence of phenylpyruvic acid formed by the oxidative deamination of phenylalanine, a further attempt to demonstrate this relationship was made by a study of the influence on the urinary picture of a derivative of phenylalanine in which oxidative deamination was prevented by the "blocking" of the α -amino group. It has been shown that the α -ureido derivatives of amino acids are stable in the animal organism and do not readily yield their nitrogen as urea (11, 12). The α -ureido derivative of phenylalanine, α -ureido- β -phenylpropionic acid, was prepared by the reaction of phenylalanine and potassium cyanate according to the usual method. This α -ureidophenylpropionic acid was fed or injected in a series of experiments. As shown in Table IV, no rise in the phenols of either blood or urine resulted after the administration of this compound. Although the excretion of total nitrogen increased, there was little change in the urea nitrogen excretion on the experimental day. The extra nitrogen, not urea nitrogen, excreted on this day was 0.446 gm., corresponding to a recovery of slightly more than 80 per cent of the nitrogen of the α -ureidophenylpropionic acid injected. Similar results were obtained after oral administration. This would indicate that the "blocking" of the amino group of phenylalanine is able to prevent deamination and that in the absence of deamination as the first step in the intermediary reactions of phenylalanine, the changes in the urinary picture, which we believe to be due to the presence of phenylpyruvic

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acid, are not to be observed. No evidence is offered by these experiments to support the theory that phenolic derivatives may be formed by oxidation of the benzene nucleus of phenylalanine in the absence of oxidative deamination.

Embden and Baldes (13) perfused phenylalanine through the liver of the dog and were able to isolate tyrosine in the perfusion

TABLE IV
Influence of α -Ureidophenylpropionic Acid on the Urinary Excretion
Rabbit; weight, 3.45 kilos.

Day of experiment	Length of period	Urine						Blood*
		Creatinine	Total N	Urea + ammonia N	Amino N	Phenols		Total phenol
						Total	Free	
	hrs.	gm.	gm.	gm.	gm.	gm.	gm.	mg.
1	6	0.036	0.279	0.191	0.002	0.017	0.012	
	6	0.040	0.337	0.253	0.003	0.017	0.013	
	12	0.076	0.607	0.486	0.005	0.031	0.020	2.5
	24	0.152	1.223	0.930	0.010	0.065	0.045	
2†								2.5‡
	6	0.045	0.671	0.233	0.003	0.017	0.009	2.5‡
	6	0.045	0.347	0.187	0.003	0.016	0.009	2.5
	12	0.089	0.678	0.536	0.006	0.026	0.015	2.5
	24	0.179	1.696	0.956	0.012	0.059	0.033	
3	6	0.048	0.380	0.290	0.004	0.014	0.008	2.5
	6	0.047	0.335	0.244	0.003	0.016	0.010	
	12	0.081	0.636	0.522	0.005	0.028	0.018	
	24	0.176	1.351	1.056	0.012	0.058	0.036	

* The results are given in mg. per 100 cc.

† At the beginning of this period 3.970 gm. of α -ureidophenylpropionic acid (nitrogen, 0.534 gm.) were injected subcutaneously as the sodium salt.

‡ Blood was taken 3 hours after the injection.

fluid. They postulated that the formation of phenylpyruvic acid was not the primary reaction in the catabolism of phenylalanine but that direct oxidation of the benzene nucleus either preceded or occurred simultaneously with oxidative deamination. If oxidation to tyrosine occurred, the further reactions of intermediary

metabolism of the two amino acids should be the same. Dakin (14) in a test of this hypothesis in the living organism (rabbits) failed to demonstrate a conversion of phenylalanine to tyrosine or other phenolic derivatives and was unable to support the theory of a conversion of phenylalanine exclusively to tyrosine. Kotake and his coworkers (15), in experiments in which large amounts of phenylalanine (9 gm. daily to rabbits of 2.55 and 2.40 kilos) were fed, obtained evidence of the elimination of *p*-hydroxyphenylpyruvic acid in the urine. This substance might have been formed by the oxidation of the phenylalanine to tyrosine, with subsequent oxidative deamination or by oxidative deamination of phenylalanine followed by oxidation of the phenylpyruvic acid formed to the hydroxy derivative. Our experiments, in which the amounts of amino acids used were much smaller, fail to support this theory of a common metabolic path for the two aromatic amino acids. *p*-Hydroxyphenylpyruvic acid, as does phenylpyruvic acid, reacts with ferric chloride to give a bluish green color with ferric chloride, but we have never observed this reaction in the urine after tyrosine feeding even though the Folin-Denis phenol method indicated an increased content of phenolic substances. The reactions of the urine after the administration of tyrosine were not the same as those observed after phenylalanine. Moreover, in unpublished experiments from this laboratory (10), in which a different method of attack on this problem was used, we have never been able to demonstrate the presence of *p*-hydroxyphenylpyruvic acid in the urine after feeding phenylalanine to rabbits. A further report of these studies will be presented at a later date.

Our data do not rule out the presence of either *p*-hydroxyphenyllactic or phenyllactic acid in the urines of the experimental days. Phenyllactic acid gives a faint yellow-green color with dilute ferric chloride solutions, but the color is distinctly different from the intense blue-green obtained with phenylpyruvic acid. Phenyllactic acid does not reduce either the diazotized *p*-nitroaniline used by Theis and Benedict or the Folin-Denis phenol reagent as does phenylpyruvic acid, the colors obtained in these reactions with phenyllactic acid being only slightly more intense than those of the blank determinations. The reactions observed in our experiments cannot therefore be due to the presence of phenyllactic acid. *p*-Hydroxyphenyllactic acid, on the other

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hand, by virtue of its hydroxyphenyl group, should reduce the phenol reagent. The increased phenol content of the urine after tyrosine feeding may be due to the presence of this acid rather than to the presence of more simple phenolic derivatives. We believe that the absence of *p*-hydroxyphenylpyruvic acid is indicated by the negative ferric chloride test and the failure of the urine to show an increased chromogenic value with picric acid and alkali. It is stated that, "in the case of phenylalanine the most probable primary product of deamination appears to be an α -hydroxy acid, while in the case of tyrosine it appears to be an α -ketonic acid" (Mitchell and Hamilton (9)). Our data would appear to demonstrate the presence of an α -keto derivative in the urine as a product of the metabolism of phenylalanine and the absence of such a derivative in the urine as a product of the metabolism of tyrosine. If the α -keto acid were formed from tyrosine it is, of course, possible that further oxidation has occurred.

SUMMARY

1. After oral or subcutaneous administration of phenylalanine to rabbits in moderate amounts (0.91 gm. per kilo of body weight), the analytical data and reactions of the urine indicated the excretion of a significant amount of phenylpyruvic acid. After the similar administration of phenylpyruvic acid, evidence of the excretion of considerable amounts of the unchanged acid was obtained. The complete oxidation of the aromatic nucleus of phenylalanine appears to occur less readily than is usually assumed.

2. α -Ureido- β -phenylpropionic acid, a derivative of phenylalanine in which the amino group is "blocked," was not oxidized in the organism of the rabbit. It is believed that oxidative deamination is a necessary step in the intermediary metabolism of phenylalanine prior to the oxidation or disruption of the benzene nucleus.

3. No evidence of the excretion of *p*-hydroxyphenylpyruvic acid after the administration of tyrosine was obtained.

4. The experimental data do not support the theory (13) that oxidation of the benzene nucleus with the formation of tyrosine is the primary reaction in the intermediary metabolism of phenylalanine.

5. A study of the distribution of the non-protein nitrogen of the blood after administration of tyrosine and phenylalanine is reported.

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A QUINHYDRONE-COLLODION ELECTRODE OF SPECIAL APPLICABILITY IN EXPERIMENTAL PATHOLOGY

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With the exception of the glass electrode, all the contemporary electrometric methods of measuring hydrogen ion activities require a certain amount of manipulation and alteration of the fluid, either by saturating it with a gas (hydrogen electrode) or with some one of the substances forming an oxidation-reduction system developing a potential as a function of the hydrogen ion activity (quinhydrone electrode). Due to this intimate admixture of the substances of the fluid under examination and those introduced for the purpose of establishing an electrode system, definite and important limitations are created in the use of the methods. The hydrogen electrode is unsatisfactory in the presence of agents that are even weakly oxidizing, since secondary oxidation-reduction potentials may be set up to confuse the real measurement. Furthermore, elaborate precautions are necessary to prevent the removal of the dissolved gases where these are important in establishing the reaction. With quinhydrone, the dissociated quinone and hydroquinone may be oxidized or reduced so that the concentration ratio of these two substances is no longer unity, a fundamental condition in the application of this electrode.

Therefore, it seemed important to devise some means whereby hydrogen ion activities could be measured without altering or disturbing the fluid being studied. This can be accomplished by segregating a very small portion of the fluid from the main body by the use of a semipermeable membrane across which equilibrium can be established. If the segregated portion is confined in a small collodion sac and the contained fluid kept saturated with quinhydrone, then a platinum or gold wire placed inside the sac will acquire a potential which will be a function of the hydrogen

ion activity within the sac, provided that the membrane is impermeable to quinone and hydroquinone or that these two substances diffuse through the membrane at equal rates.

If, then, a reference half-cell is connected with the fluid outside the sac and the potential difference between the half-cell and the platinum wire within the sac is measured, this value will be the sum of all the potential differences in the system: at the platinum wire, at the membrane, and all the remaining interfaces of the system including those within the half-cell used as a reference. Since the interfaces within the reference half-cell remain constant in character, the sum of these potentials constitutes the total potential and need not be further considered. The half-cell may be connected with the solution by a saturated KCl bridge so that no significant diffusion potential is developed.

There remains but one potential which must be eliminated before a measurement over the entire cell will give the value at the electrode within the sac. This potential results from the unequal dispersion of ions due to relative semipermeability of the membrane. Its value may be calculated from considerations advanced by Donnan (1) provided that the system is simple and readily subject to exact analysis and also that the membrane is impermeable to one ion species. In general, the solutions of biological interest are very complex so that the analytical method of Donnan is inapplicable. Further, the collodion membrane is not strictly impermeable; rather it serves to check the diffusion of quinhydrone into the solution. However, it is possible to demonstrate that in solutions containing appreciable quantities of electrolyte (0.01 M or greater), no significant membrane potential arises.

In all equations pertaining to the quinhydrone electrode, there are certain basic assumptions which must not be violated. The complete equation for the quinone-hydroquinone system is (2)

$$(1) \quad E = \frac{RT}{F} \ln \frac{(C_6 O_2 H_4) (H^+)}{(C_6 O_2 H_2)} + C$$

where the parentheses indicate activities rather than concentrations and C is an additive constant to be determined. This equation is derived under the limiting assumption that the primary as well as the secondary ions of the hydroquinone form a negligible proportion of the total hydroquinone. This assumption is valid

below a pH of approximately 8.5; for this reason this electrode system is ill adapted to solutions more alkaline than 8.5 due to the greater dissociation.

By assigning fixed values to the concentrations of quinone and hydroquinone, the activity ratio becomes practically constant and the equation is greatly simplified. This is done in practice by saturating the solution with quinhydrone which in aqueous solution dissociates into 1 molecule each of quinone and hydroquinone (3).

The equation may now be written:

$$(2) \quad E = -\frac{RT}{F} \ln \frac{1}{(H^+)} + E_q$$

where E_q is the value of E between the reference half-cell and the quinhydrone electrode when the solution has unit activity with

TABLE I
Value of θ at Various Temperatures

<i>t</i>	θ	<i>t</i>	θ
°C.		°C.	
17	0.0575	29	0.0599
19	0.0579	31	0.0603
21	0.0583	33	0.0607
23	0.0587	35	0.0611
25	0.0591	37	0.0615
27	0.0595	39	0.0619

respect to hydrogen ions. Changing to the common logarithms so as to use the hydrogen ion exponent scale, the equation becomes

$$(3) \quad \text{pH} = \frac{E_q - E}{\theta}$$

where θ is $\frac{RT}{F} \log_e 10$. The value of this factor is given in Table I (4) for various temperatures, t .

Construction of Electrode

A small piece of platinum wire is sealed in the end of a small glass tube 10 to 20 cm. in length. Within the tube is placed an amount of mercury sufficient to serve as a contact with a fine bare

copper wire as a conductor between the mercury and the exterior (Fig. 1).

The platinum is plated with a thin deposit of gold from a solution of gold chloride, dried, and heated with the blast lamp until the gold fuses. A better plate is established by depositing the gold from a solution of gold cyanide and polishing with rouge. This establishes a smooth gold surface with a minimum of labor. While pure polished platinum alone is entirely satisfactory for these electrodes, the surfacing with pure gold obviates any difficulties that might arise from impure platinum or a roughened surface.

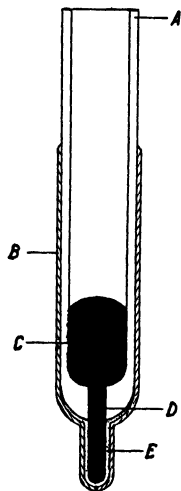


FIG. 1. Detail of quinhydrone-collodion electrode. *A*, glass tube; *B*, collodion membrane; *C*, mercury contact; *D*, platinum wire, the exterior surface being gold-plated; *E*, deposit of quinhydrone.

The electrode is prepared by repeatedly dipping the wire into a saturated solution of quinhydrone in ether, time being allowed for drying after each immersion. Quinhydrone is deposited in fine needles so that gradually a dense deposit is established. Without allowing the quinhydrone to dry entirely, the electrode is dipped into a 4 to 6 per cent collodion and, held horizontally, is slowly rotated while the collodion dries. A second dipping is advisable to establish a good membrane. The electrode is now ready for use.

Equilibrium is reached most satisfactorily when the fluid being examined moves slowly past the electrode. This necessitates some type of electrode vessel which will permit a continuous flow. Such a vessel also is readily incorporated in a closed system so that fluids may be examined under constant and known partial pressures of gases. Such an electrode vessel, especially adapted to a closed system, is shown in Fig. 2. Connection to the reference half-cell is made by means of a side arm and rubber tubing. A T inserted in the rubber tubing may be used to vent the entrapped air.

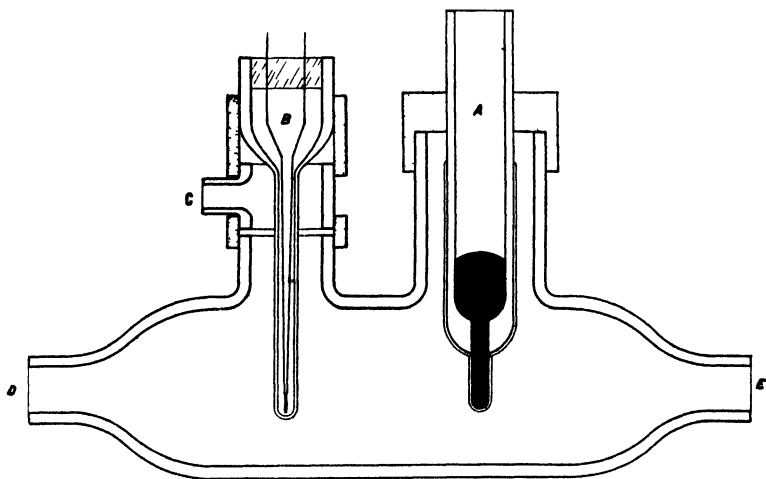


FIG. 2. Electrode vessel, closed type. A, quinhydrone-collodion electrode; B, copper-constantan thermocouple; C, side-arm for junction with calomel cell; D and E, fluid passageway.

Tight joints are made with short pieces of soft rubber tubing as indicated.

If the fluid examined is at a temperature different from that of the room, a thermocouple may be employed to measure the exact temperature at the electrode. A copper-constantan thermocouple is placed in a thin walled glass tube closed at one end. This passes into the electrode vessel through the same side arm used for the reference half-cell connection. The cold junction may be placed in any type of constant temperature bath desired. A mixture of ice and water furnishes one of the most convenient and

reliable cold junction baths. With use of a double throw switch, the potentiometer may be used for measuring the temperature a few seconds after the E. M. F. of the electrode is determined. This is particularly important if the electrode vessel is used in the vascular system of a living animal for direct determinations of the pH of the blood.

TABLE II

Performance of Electrodes Placed Simultaneously in One Electrode Vessel

Electrode A Thin deposit of quinhydrone Thin collodion membrane		Electrode B Medium deposit of quinhydrone Medium collodion membrane		Electrode C Heavy deposit of quinhydrone Thick collodion membrane		Electrode D Medium deposit of quinhydrone Medium collodion membrane	
Time	E. M. F.	Time	E. M. F.	Time	E. M. F.	Time	E. M. F.
<i>min.</i>	<i>volt</i>	<i>min.</i>	<i>volt</i>	<i>min.</i>	<i>volt</i>	<i>min.</i>	<i>volt</i>
3	0.0230	3	0.0231	6	0.0500	5	0.0227
10	0.0231	9	0.0230	10	0.0340	8	0.0230
Electrode bare at 18'		14	0.0232	12	0.0295	10	0.0230
		Electrode bare at 20'		17	0.0257	15	0.0233
				22	0.0242	Electrode partly bare at 20'	
				28	0.0236		
				35	0.0237		
				Quinhydrone still remaining			

Mean E. M. F. = 0.0234 $E_0 = 0.4529$

The membranes were then stripped from the electrodes and the buffer solution saturated with quinhydrone. The electrodes were replaced so that the usual quinhydrone system was established.

5	0.0234	5	0.0233	5	0.0233	5	0.0233
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Mean E. M. F. = 0.0233

Results satisfactory for most purposes may be obtained without any provision for securing a flow. The electrode is merely suspended in a small vessel containing the fluid. Connection is made with the reference half-cell and the E. M. F. measured.

Performance of Electrode

To determine the magnitude of the potential established by the introduction of the membrane, four electrodes were prepared. Of

these, Electrodes A and B were surfaced with gold deposited from AuCl_3 solution and the deposit then fused in the blast lamp; Electrodes C and D were plated from a solution of gold cyanide and the plated surface given a high polish with Turkish emery followed by rouge. Phosphate buffer solution, pH 7.30 as determined by the hydrogen electrode, was used and time was measured in minutes from the instant of placing the electrode in the solution. The temperature was 23° . A saturated calomel cell was used as the reference half-cell with a saturated KCl bridge. The four electrodes were placed simultaneously in one electrode vessel which had previously been rinsed several times with the buffer solution. The results are shown in Table II.

From this it appears that variations in the electrode coatings affect the rapidity with which equilibrium is approached but do not appreciably affect the equilibrium value itself, very thin deposits giving rapid equilibration but short life. Furthermore, in such buffer solutions no significant membrane potential is found as the change in E.M.F. on adding quinhydrone to the solution in the usual way is no greater than the variation among the electrodes themselves and this is of a relatively small order. An error of 0.0005 volt in the measured potential difference entails a variation of 0.008 in the calculated pH.

A second source of membrane potential lies in the possible presence of charged colloidal substances in the solution. A simple solution, functioning with both hydrogen and quinhydrone electrodes, was made of 1 per cent gelatin, primary and secondary phosphates to form a buffer system, and KCl to give a total electrolyte concentration of 0.06 M. Two such solutions were prepared. After the quinhydrone-collodion electrodes were allowed to reach steady values, the membranes were removed, the solutions saturated with quinhydrone, and the electrodes returned. The results are shown in Table III.

Here again no significant membrane potential is encountered. These observations are in accord with the conclusions of Donnan; namely, that addition of electrolytes lowers the membrane potential. Michaelis (4) has shown that in solutions where the electrolyte concentration exceeds 0.01 M, and the charged colloidal ion is approximately 0.1 M, the membrane potentials possible are negligible in practical work.

With the quinhydrone-collodion electrode, a further condition must be considered before equation (3) can be accepted; that is, the quinone and hydroquinone resulting from the dissociation of quinhydrone must diffuse through the membrane at approximately equal rates so that the quinone-hydroquinone ratio shall not be appreciably disturbed from unity. Ideally it would be desirable

TABLE III
Results Obtained on Buffered Gelatin Solutions

	Solution A	Solution B
Hydrogen electrode, pH.....	5.67	5.64
Quinhydrone-collodion electrode, <i>volt.</i>	0.1215	0.1227
Removed membrane and saturated solution with quinhydrone, <i>volt.</i>	0.1217	0.1228
E_q	0.4546	0.4541

TABLE IV
Diffusibility of Quinone and Hydroquinone Resulting from Dissociation of Quinhydrone

Time	Electrode 1	Electrode 2
<i>min.</i>	<i>volt</i>	<i>volt</i>
3	0.1544	
4		0.1528
5		0.1521
6	0.1519	
10	0.1525	
15	0.1526	
16		0.1521
28		0.1527
29	0.1525	

$$E_q = 0.4536$$

to secure impermeability with respect to these molecules, but this is not possible. Additional evidence of this equal diffusibility is found in the following experiment.

Two quinhydrone-collodion electrodes were placed in an electrode vessel similar to Fig. 2. Continuous flow of a phosphate buffer solution of pH 5.13 was secured. A saturated calomel half-

cell was used as reference. The room temperature was 22°. Time was measured in minutes from the moment of insertion of electrodes into the vessel. The determinations are given in Table IV.

At no time after the first 4 minutes did the electrodes differ by more than 0.0005 volt. Furthermore, the individual electrodes varied only 0.0006 volt in 25 minutes, the change in each being of the same magnitude and direction. This performance can be explained only on the basis that the quinone and hydroquinone diffuse through the membrane at equal rates so that in effect there is merely a loss of solid quinhydrone.

A more complicated system, and one well adapted to measurement with the hydrogen electrode, is urine. The pH of specimens allowed to stand at room temperature for several hours was determined by means of the hydrogen electrode. Two quinhydrone electrodes were then placed in each of the samples used for the hydrogen electrode measurement. In no instance did the two electrodes vary by more than 0.0005 volt after reaching steady values. Mean values for E_q of 0.4472, 0.4482, and 0.4506 were obtained on successive separate specimens of pH 5.47, 6.14, and 6.14 respectively, the specific gravity being variable. Urine samples saturated with quinhydrone in the usual way showed fair stability of potentials, drifting approximately 1 millivolt in 2 hours.

A still more complicated system, less readily adapted to measurement with the hydrogen electrode, is blood. Here the passage of hydrogen to secure saturation results in a profound disturbance of all gaseous equilibria. With the quinhydrone system, the potential drifts rather rapidly and in whole blood, the drift is sufficient to invalidate the results. With serum or plasma, more stable values are obtained although even these show a marked drifting tendency so that various investigators have arbitrarily taken their readings at set time intervals after introducing the quinhydrone. This method gives results that are consistently reproducible, but there is grave doubt that the system has reached a true equilibrium in most cases.

The quinhydrone-collodion electrode was found to give readily reproducible values in whole blood and blood plasma, after about 10 minutes equilibration. Fig. 3 shows the potential as a time function of a single electrode in whole rabbit blood at 23°, the

blood being allowed to move past the electrode. The curve is drawn through all the plotted points and is transcendental in character. Paired electrodes in ten specimens gave agreement to

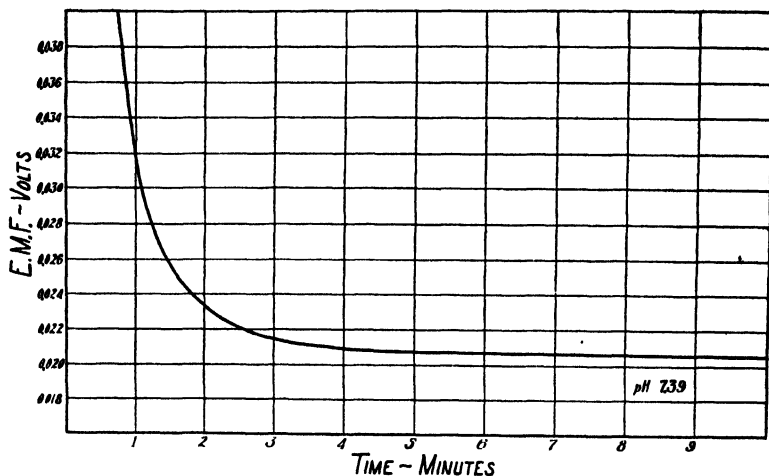


FIG. 3. Electrode in whole rabbit blood

within 1 millivolt. In order to arrive at the correct value of E_q , a specimen of old blood, completely hemolyzed, was placed in the electrode vessel and hydrogen passed until steady values were

TABLE V
Results with Hemolyzed Blood

Hydrogen electrode	Quinhydrone-collodion electrodes					
	Electrode 1		Electrode 2		Electrode 3	
	Time	E M F.	Time	E M F.	Time	E. M. F.
	min.	volt	min	volt	min.	volt
$E = 0.7216$ volt	20	0.0249	20	0.0237	15	0.0237
$E_0 = 0.2457$ "			Mean = 0.0240			
pH = 8.10			$E_q = 0.4519$			

obtained with the hydrogen electrode. Into the thoroughly reduced material, quinhydrone-collodion electrodes were placed. The results are shown in Table V.

Thus, it is seen that while values with the quinhydrone-collodion system are reproducible, the values of E_q in solutions of varying types differ to an appreciable degree. This cannot be neglected in careful work if the second decimal place of pH is considered significant. In variations of this type, the so called "salt effect" plays an important part, the dissolved substances affecting the activities of the components of the quinhydrone system. Therefore, it is a matter of some importance that the quinhydrone system be standardized by the use of a system similar to that being studied and which, in addition, can be exactly evaluated by means of the hydrogen electrode. In this way, the ultimate comparison is with the hydrogen electrode which is presumably not affected to any great extent by dissolved substances provided they do not enter into secondary reactions.

In this work, all buffer solutions have been standardized by the use of the hydrogen electrode, E_0 being determined by setting up a cell containing 0.0103 N HCl, the activity coefficient of this concentration of acid being taken as 0.924 (5) at 23° so that the pH of this solution is 2.0218. A saturated calomel cell was used as a reference. In every experiment the assembled cell was standardized with a standard buffer solution.

SUMMARY

A quinhydrone-collodion electrode is described which allows the determination of the hydrogen ion activity of a solution without alteration of the solution itself. It is stable in biologic fluids which are unsuitable for study by other methods.

The electrode is especially adapted to use in a closed system in which definite partial pressures of dissolved gases may be maintained.

The electromotive force developed is within 1 millivolt of its final value in 3 to 10 minutes, depending on the thickness of the membrane and of the quinhydrone deposit.

Ease of preparation and reproducibility, together with precision of potentials, characterize the electrode.

The value of E_q should be determined by means of solutions similar or identical in composition with those studied and whose pH may be determined directly with the hydrogen electrode.

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STUDIES ON THE SPONTANEOUS OXIDATION OF CYSTEINE

III. THE METHOD OF ACTION OF CYANIDES AND CYSTINE ON CYSTEINE OXIDATION

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INTRODUCTION

Bernard (1), in 1857, investigated the physiological effect of cyanide and the succeeding contribution of Gaehtgens (2), Gelpert (3), and many others indicate that in some way or other exposure of living organisms to adequate concentrations of cyanide is followed by a reduction in the O_2 consumption and CO_2 production. Cyanide is therefore considered as a respiratory poison. That cyanides and nitriles inhibit the spontaneous oxidation of cysteine solutions was discovered by Mathews and Walker (4) who considered the possibility that it inhibited by uniting with iron in the solutions, but discarded this possibility in favor of a direct union of cyanide with cysteine since they believed that their preparations were free from iron. Warburg (5), Wieland (6), and Thunberg (7) make extensive use of this cyanide depression action on cysteine oxidation in support of their theories of biological oxidation-reduction.

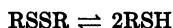
Warburg believes that the cyanides inhibit respiration by combining with the iron which, in combination with cysteine, he believes to be the respiratory enzyme responsible for cellular oxidations. He concludes that intracellular iron combines in some unknown chemical way with the cyanide.

Voegtlin (8), opposing this theory, states that Warburg failed to perform the crucial experiment; *i. e.*, he did not demonstrate

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that the inhibition of respiration produced by threshold concentrations of cyanide can be overcome by equivalent amounts of iron salts. He found that previous injections of iron salts into rats failed to antagonize minimum lethal doses of sodium cyanide. He did note, however, that cystine, cysteine, or glutathione did possess such action and, when injected in the proportion of 5 parts of the sulfhydryl compound to 1 part of cyanide, the animal was protected.

From these results he concluded that the inhibiting action of the cyanides on respiration is due not to a conversion of intracellular iron to an inactive complex substance but rather to a disturbance in the equilibrium between reduced and oxidized sulfur compounds as represented in the following relation.



He believed, as has previously been suggested by Professor Mathews, that the toxic action of cyanides is due to chemical reaction which takes place between the cyanide and the cystine sulfur of tissues. Making use of polarimetric measurements on a solution of cystine and varying amounts of cyanide, he found that in such solutions the cystine was reduced to cysteine as indicated by specific rotations of small negative values. Then on aeration the specific rotation again reached a high negative figure which indicated complete reversion to cystine. Hence, he assumed that the reaction taking place is that of reduction of cystine to cysteine and the subsequent conversion to the oxidized form by aeration. The possibility that NaCN is oxidized to NaCNS is practically ruled out, according to his evidence, as such a change would involve the splitting off of S from the cystine. The aeration experiments indicate that the sulfur is not split off.

He concluded that the cyanide is converted into the cyanate according to the following equation,



which does not involve the participation of molecular O_2 and is therefore applicable to anaerobic oxidation.

However, much evidence is supplied by Bodansky and Levy (9) and others confirming much earlier work to show that cyanides

and nitriles are converted into the thiocyanates within the body. The former (10) has shown recently that in alkaline solutions cystine converts cyanide into the thiocyanate *in vitro* to the extent of as much as 60 per cent, but in neutral solution the reaction occurs only to a very slight extent. Testing such solutions by a special method he failed to detect the slightest amount of NaCNO. It was also shown that the oral administration of KCN produced a marked increase of the thiocyanate content of the saliva, which he believed argues for the physiological conversion of cyanides into thiocyanate.

Using the procedure of Mauthner (11), he isolated from alkaline cyanide-cystine mixtures a substance which on analysis proved to be α -amino- β -sulfocyanopropionic acid. This result suggests that at least a portion of the thiocyanate is in organic combination as the compound mentioned above. This conversion is expressed by



thus confirming Pulewka and Winzer (12) who report that in the reaction between cystine and cyanide a molecule of cysteine is formed for each molecule of cystine, as determined by iodometric titration.

Dixon and Elliot (13), attempting to determine the extent of cyanide inhibition on the oxygen uptake of various tissues, found the average maximum inhibition reached to be about 60 per cent. In no case was the respiration completely inhibited by cyanides but in some tissues and especially in yeast, the inhibition reached 90 per cent of the normal rate of oxygen uptake. In the majority of cases 0.001 M concentration of cyanide was sufficient to produce the maximum degree of inhibition obtainable.

From these results they conclude that the respiration of animal tissues is made up of two parts. One, accounting for about two-thirds of the total, is due to systems poisoned by cyanides, the other one-third is due to systems which are stable to cyanides. The same authors believe that cyanide-stable systems such as the xanthine oxidase may contribute to the cyanide-stable part of respiration. However, xanthine oxidase cannot account for the whole of this part, since Morgan has shown that it is absolutely absent from muscle, yet it is likely that there are a number of other systems of this type.

EXPERIMENTAL

Although it has been shown that cyanide inhibits cysteine oxidation in much the same manner as it does tissue oxidation, yet in no instance during the experiments of the author has the inhibition been complete. Even though it has been shown by Sakuma (14), Harrison (15), and the author (16) that pure iron-free cysteine oxidizes only with extreme slowness when compared to that catalyzed by metals, still no proof exists to show that cysteine is not autoxidizable. Hence, it is very likely that cysteine itself may be a component of the cyanide-stable system.

Harrison, who obtained oxidation rates for his pure cysteine hydrochloride as low as 2.32 c.mm. of O_2 per hour per 10 mg. of $RSH \cdot HCl$, was able to inhibit oxidation only to the extent of a 1.86 c.mm. of O_2 per hour uptake in one case, and a 2.94 c.mm. of O_2 per hour uptake in another. In both experiments cyanide was present in 0.001 M concentration.

Since cyanide is such a powerful inhibitor of iron catalysis it would certainly seem reasonable to believe that if the residual oxidation were due to iron, then cyanide should completely inhibit oxidation. If there were present in a 10 mg. sample of cysteine HCl about 0.000044 mg. of Fe , as was shown in a previous paper (12) to be the amount necessary to account for this residual oxidation if we are to assume that iron is the only active substance, then if the solution contained cyanide to the extent of 0.001 M concentration, there would be present 1,000,000 molecules of cyanide for every molecule of iron.

A series of experiments was carried out to determine whether cyanide could depress the oxidation rate of a specially purified cysteine hydrochloride prepared according to a method described by the author (17), by means of which cysteine hydrochloride was obtained which contained less than 1 part of iron in 20 million parts of the cysteine hydrochloride. The lack of inhibiting action in the pure cysteine is shown in Table I which gives definite proof that cyanides do not inhibit the oxidation of cysteine when completely freed from iron.

That the oxygen uptake in my preparation could not be due to the oxidation of HCN to $HCNO$ by atmospheric oxidation was ruled out by the results of a previous experiment in which it was shown that a pure 0.01 M HCN solution took up absolutely no

measurable quantity of O_2 when measured in the Barcroft apparatus under identical conditions.

In order to show that the cyanide would inhibit the oxidation if it were due to iron, experiments were performed in which oxida-

TABLE I
Effect of Cyanide on Oxidation of Cysteine

pH	Temperature	O_2 uptake of 10 mg. RSH · HCl per hr.	HCN added to make final concentration	O_2 uptake after cyanide addition
	°C.	c.mm.	M	c.mm.
7.4	22	3.2	0.001	2.8
7.6	22.5	3.3	0.005	2.4
7.4	24.0	2.8	0.04	3.0
7.6	23.0		0.005	2.2
7.6	22.5		0.01	2.3
7.4	24		0.001	2.0
7.6	25		0.01	3.1
Average uptake				2.5

It has been shown in a previous paper that pure iron-free cysteine oxidizes at an average rate of about 2.2 c.mm. of oxygen per hour per 10 mg. sample (16). Oxidation was measured by means of a Barcroft micro respirometer.

TABLE II
Effect of Cyanides on Iron Catalysis

Original O_2 uptake	Fe added as $FeCl_3$	Catalyzed O_2 uptake	HCN added to make solution	Cyanide-inhibited O_2 uptake
c.mm.	mg.	c.mm.	M	c.mm.
2.5	0.0010	57.0	0.01	1.9
3.2	0.0010	50.3	0.01	3.0
1.9	0.0005	21.2	0.001	2.2

The uptake rates are expressed in terms of c.mm. of oxygen per hour per 10 mg. sample of cysteine hydrochloride.

tion rates were determined for the pure substance. Iron was then added and the increased oxygen uptake noted. When cyanide is added to these catalyzed oxidations the rates are inhibited only to the extent of catalysis by the added iron. In other words, the oxidation now goes on at the original rate. It may be noted from

Table II that the inhibited rates are almost identical to oxidation rates before the iron addition.

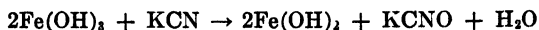
That the cyanide in solution is relatively stable for a fairly long period of time was shown in an experiment in which the original uptake was 1.8 c.mm. of O_2 per hour per 10 mg. of cysteine hydrochloride. Enough HCN solution was then added to make the final concentration 0.001 M. The oxidation continued at approximately the same rate for 10 hours, after which 0.001 mg. of Fe was added and readings again taken. The added iron failed to increase the oxidation showing that the added iron was immediately converted into an iron-cyanide complex in which form it could not act catalytically.

In conclusion, pure cysteine oxidizes at approximately the same rate in the presence of varying amounts of cyanide as in its absence. Cyanides do not inhibit completely the oxidation even in the presence of iron and reduce the rate of uptake only to the extent of that catalyzed by iron. Cysteine, catalyzed by iron and oxidizing at a high rate, takes up oxygen only at a rate equal to that of cysteine alone after cyanide is added. From these experiments it is evident that cyanide acts by converting iron into a non-catalytic form and exerts no influence on pure cysteine.

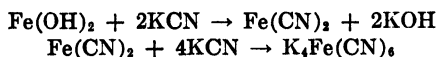
Action of Cyanides on Iron Solutions

Since iron in a cysteine solution is so rapidly converted into a complex compound in which form it no longer exerts any catalytic effect, a study was made of the nature of the formation of a complex iron-cyanide compound. When KCN solution is added to a ferrous ammonium sulfate solution a dirty blue precipitate forms which on standing settles to the bottom of the flask. This consists of ferrous hydroxide which partially oxidizes to the ferric state. The supernatant solution on neutralization and the subsequent addition of ferric chloride gives a heavy blue precipitate of the well known Prussian blue, indicating the formation of $K_4Fe(CN)_6$. When ferric chloride is added to KCN there is formed a heavy brown precipitate consisting of ferric hydroxide. The yellow supernatant liquid when tested with a ferrous salt gives the typical Turnbull's blue which confirms the presence of $K_4Fe(CN)_6$. However, when a ferric salt instead is added to this same solution a heavy blue precipitate of Prussian blue is formed. This means

that in addition to $K_3Fe(CN)_6$ there is also $K_4Fe(CN)_6$ present. It is very improbable that KCN can reduce $K_3Fe(CN)_6$ to $K_4Fe(CN)_6$. The formation of the $K_4Fe(CN)_6$ is no doubt due to an interaction between the cyanide and some $Fe(OH)_2$ formed as a result of a reduction of the $Fe(OH)_3$ by KCN according to the following equation.



Then the $Fe(OH)_2$ combines with KCN to form $K_4Fe(CN)_6$ according to the reactions,



From the results of these reactions, which take place also at neutral reaction, it is quite evident that this complex iron-cyanide compound which Warburg assumes to be formed may in reality be either $K_4Fe(CN)_6$ or a combination of $K_4Fe(CN)_6$ and cysteine. If the iron is assumed to be bound up in this manner then it must be shown that iron added in the form of the complex cyanide does not catalyze cysteine oxidation.

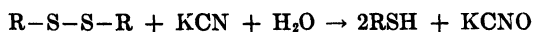
When 0.001 mg. of iron is added to cysteine in the form of either $K_3Fe(CN)_6$ or $K_4Fe(CN)_6$ the oxidation rate is only slightly greater than that of cysteine alone averaging 5.6 c.mm. of O_2 per hour whereas cysteine oxidizes at the average rate of 2.2 c.mm. of O_2 per hour. An equivalent amount of iron added in ionic form as $FeCl_3$ caused an increased rate of 50 to 60 c.cm. of O_2 per hour. The slight increase over the oxidation of cysteine alone may be accounted for by the traces of free iron which the complex cyanide contained. It is quite evident from these experiments that the iron in the complex cyanide cannot function as a catalyst.

If ferrous or ferric salt is added to a neutral cysteine solution containing KCN, $K_4Fe(CN)_6$ is formed as detected by the formation of Prussian blue when a small amount of ferric salt is added. When ferric salt is added to a neutral cysteine solution it is immediately reduced to the ferrous state. A consideration of the ease with which iron is converted into the complex cyanide on the addition of KCN both in the presence and absence of cysteine, points to the possibility that this reaction may represent the inhibiting action of cyanides on iron catalysis. The inability

of $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ to act catalytically on the cysteine oxidation lends confirmation to this possibility.

Effect of Cystine on the Autoxidation of Cysteine

As had been mentioned in a previous section Abderhalden and Wertheimer (18) observed a definite inhibiting action of cyanides on the oxidation of their samples of cysteine hydrochloride which they believed to be iron-free. They attributed this inhibiting effect to the action of cyanides on the cystine rather than the action on any trace of iron which might have been present. Dixon and Tunnicliffe (19) claim that cystine greatly accelerates the oxidation of cysteine by atmospheric oxygen, and that the maximum reactivity is reached when a ratio of 2.5 parts of the disulfide to 1 part of cysteine exists. Hence, Abderhalden and Wertheimer attribute the slight oxidizability of the cysteine to a contamination of cystine which they believed to be present in their samples. Since cyanide is able to reduce cystine to cysteine, the inhibition of the oxidation of cysteine by cyanide is due, according to them, to the removal of the catalytic cystine thus,



However, no such action of cystine was observed in experiments carried out by the author. Pure iron-free cysteine hydrochloride when added to pure cysteine hydrochloride failed to increase the oxidation rates. An increased oxidation rate due to the addition of cystine could be explained only by assuming that the added cystine contained traces of iron. If cystine had such catalytic powers, then, in every experiment in which measurements were taken over long periods of time, one would observe a steady increase in oxidation rates as the conversion of cysteine to cystine proceeded, the rate reaching a maximum when 60 per cent of the cysteine had been oxidized, when, according to Dixon and Tunnicliffe, 2.5 parts of cystine would be present for every part of cysteine.

Instead of an increase in rate as would be the case if cystine were acting catalytically, one really observes a rather constant rate of oxidation and when the oxygen uptake is plotted against time the curve is linear until near the end of the oxidation when all of the cysteine has been converted into cystine.

I wish to express my gratitude for the helpful criticism and interest shown by Professor A. P. Mathews.

SUMMARY

1. The autoxidation of iron-free cystine is not inhibited by cyanides.

2. The addition of iron greatly accelerates the oxidation of pure cysteine but subsequent addition of cyanide to the catalyzed oxidation reduces its rate to the original value.

3. Potassium cyanide readily converts ferric or ferrous chloride to the corresponding potassium iron-cyanide compounds. Cyanides inhibit iron catalysis in this manner.

4. Iron added in the form of potassium ferrocyanide or potassium ferricyanide does not accelerate oxidation.

5. Pure iron-free cystine does not catalyze cysteine oxidation.

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THE RÔLE OF THE ENZYME IN THE SUCCINATE- ENZYME-FUMARATE EQUILIBRIUM

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The following is an account of an investigation into the rôle of the enzyme in the succinate-enzyme-fumarate equilibrium. The method consisted in the comparison of the value of the free energy change in this reaction obtained from oxidation-reduction potentials, with that calculated from the entropies and other physicochemical properties of succinic acid and fumaric acid.

The findings of Wishart (1), Quastel and Whetham (2), Thunberg (3), and Lehmann (4) show that the oxidation of succinic acid to fumaric acid by means of a dehydrogenase, in the presence of a reversible hydrogen acceptor such as methylene blue, satisfies the necessary criteria for thermodynamic reversibility. The equilibrium constant has been measured colorimetrically by Quastel and Whetham, with resting *Bacillus coli* as catalyst, by Thunberg, colorimetrically and electrometrically, and by Lehmann, electrometrically with an enzyme derived from horse skeletal muscle. The values obtained by the latter two workers are not concordant, and further there is some uncertainty regarding the agreement or disagreement of the potentials with the equilibrium value obtained by Quastel and Whetham.

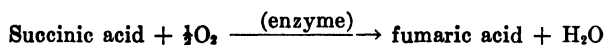
A priori, it seemed possible, apart from experimental errors, that these discrepancies might be due to variations in the nature of the enzyme employed. In other words, the enzyme is not a "perfect" catalyst, and therefore the value of the equilibrium constant (or of the oxidation-reduction potential) depends to some extent upon the nature of the enzyme employed. In this event it would be expected that the value for the standard free energy change calculated from the entropies and other physicochemical

properties of succinic acid and fumaric acid would not be the same as the experimental values, the differences being attributable to the participation of the enzymes, and varying with each enzyme.

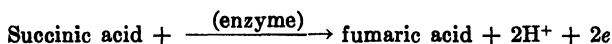
This possibility is definitely excluded, it seems, by the remarkable agreement between the calculated values and all except one of the experimental values, shown in Tables IV and V, obtained by Quastel and Whetham with "resting" *Bacillus coli* as catalyst, by Thunberg and by Lehmann with horse skeletal muscle, and by the authors with beef diaphragm and with beef heart.

This coincidence of the calculated and observed values may be taken as indicating that the enzyme promoting the oxidation of succinic acid to fumaric acid probably operates as a "perfect" catalyst, wherever it occurs, *in vivo* as well as *in vitro*.

One proviso must be made, that the reduction of the oxidizing agent, *e.g.* methylene blue, must also be perfectly reversible. It is possible that the reduction of oxygen *in vivo* is irreversible, in which case the whole reaction



is, of course, irreversible. Nevertheless it is convenient to consider the reaction,



analogous to a half-cell, which operates reversibly even *in vivo*.

This coincidence between calculated and observed values suggests one general application of the second law of thermodynamics to biological systems and is additional confirmation of the validity of the third law.

Experimental Technique and Procedure

The electrode potential measurements, yielding the results set out in Table I, were carried out with a vacuum technique, in a modified Thunberg tube depicted in Fig. 1. The principal modification consists in the attachment of a capillary tube of 0.75 mm. bore and approximately 10 cm. long, containing an agar-saturated potassium chloride bridge. When the electrode vessel is in use the end of the capillary tube dips into a vessel containing a saturated solution of potassium chloride, in communication with the

reference electrode, here a saturated calomel cell. With these vessels it is possible to carry out a number of determinations simultaneously, only one reference electrode being employed. The technical difficulty overcome was the preparation of an agar-potassium chloride bridge in glass, capable of withstanding a difference in pressure of 1 atmosphere between its two ends. After a number of trials, the following method of preparation was found to be satisfactory. Potassium chloride solution and sufficient

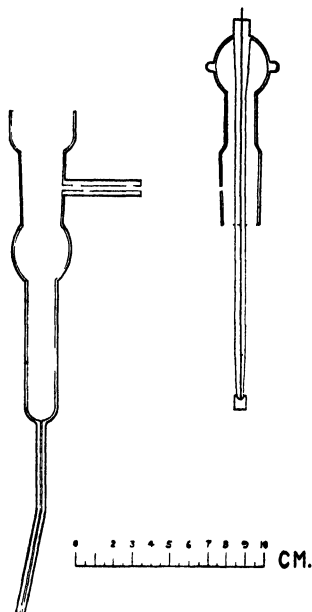


FIG 1 Modified Thunberg tube for electrode potential measurements with a vacuum technique.

dry agar to give a final concentration of 3 per cent are weighed separately, after which the agar is dissolved by heating nearly to boiling, distilled water being added from time to time so that the weight of the solution remains equal to the sum of the initial weights of the agar and salt solution. When as much as possible of the agar is dissolved, the beaker is covered and set away in an oven at 98° in which the electrode vessel is also placed. After the agar solution has become clear and free of suspended particles

of undissolved agar and of air bubbles, it is poured into a narrow test-tube which is a little taller than the length of the capillary tube of the electrode vessel. The capillary tube is then immersed in the agar solution and by slight suction quickly drawn up through the capillary, filling 1 or 2 cc. in the bottom of the vessel. The electrode vessel is then stoppered and sufficient agar solution is added to the test-tube so that the height of the solution is the same outside and inside the electrode vessel. For about an hour the temperature of the oven is maintained at 95° in order to allow the solution to wet the wall of the capillary tube. The heater of the oven is then turned off. As a rule about 4 hours elapse before the temperature falls to the jelling point of the agar. After the oven has cooled to room temperature, the outside test-tube is removed and replaced by a small vial filled with saturated potassium chloride. The agar at the bottom of the electrode vessel is scraped out with a glass stirring rod and a few cc. of salt solution are added. With both ends of the capillary tube protected in this way with salt solution when the vessel is not in use, bridges so prepared withstand a difference in pressure of 1 atmosphere even at 45° , and at 25° were used repeatedly for weeks.

The electrodes employed were mainly gold-plated platinum foil. In some of the earlier experiments platinum foil was used. The results obtained were the same with either type of electrode.

The procedure employed in the potential determinations was as follows: The specified amounts of succinate, fumarate, dye, and buffer solutions were pipetted into the electrode vessel in a cold room maintained at 2° , where the stock solutions were kept. The vessel was then stoppered and evacuated at room temperature for 1 minute, after which it was transferred to a water bath at 37° , the evacuation being continued until the solutions had been boiling for 2 minutes. After the termination of the evacuation the stopper was turned, closing the vessel, the side arm and water seal above were filled with water, and these in turn were enclosed by a layer of vaseline. The vaseline prevented any loss of water even after many hours of shaking. The evacuated vessels were transferred to an air bath maintained at 25° , at which temperature the determinations were made. It was found during the course of the investigation that more concordant results were obtained when the vessels were continually shaken throughout

the period of observation.¹ The tubes were so clamped in the shaking device that the ends of the capillary tubes containing the agar bridges dipped intermittently in and out of the saturated potassium chloride solution, in which was immersed also the end of a saturated calomel electrode.

The hydrogen ion activity determinations were made with a Moloney electrode (5)² in an air bath maintained at 25°. The reproducibility obtained with different electrodes was within ± 0.25 millivolt. The reference electrode employed for the pH determinations was also a saturated calomel half-cell, checked against the similar half-cell employed in the potential determinations. In this manner any errors due to differences between the reference cells or to liquid junctions were eliminated.

The fumarase-free enzyme solution was prepared by a modification of the method described by Lehmann. The heart and diaphragm muscle were obtained, as a rule, 2 or 3 days after the death of the animal. After removal of fat and connective tissue, the meat was passed through the finest cutter of a meat grinder, suspended in water ($\frac{1}{2}$ pound of meat in 800 cc. of suspension) and

¹ The authors are indebted to Mr. G. L. Keighley for the design and construction of the shaker employed, as well as for assistance in the working out of the method of preparing the agar bridges described above.

² The serviceability and accuracy of this type of hydrogen electrode depends upon the method of preparation of the electrode. The technique finally settled upon by the authors is the result of experience gained over a number of years by Professor H. Wasteneys and Mr. D. A. MacFadyen of the University of Toronto, and the authors. The Moloney electrode consists of 6 or 7 mm. of 1 mm. platinum wire projecting from a glass tube, and surrounded by a thread of glass forming a narrow loop extending a few mm. past the end of the wire. The electrode is first cleaned by heating in boiling aqua regia, washed with distilled water, and then heated again in boiling cleaning fluid, or immersed in hot alcoholic soda for a short time. After being washed again with distilled water it is electrolyzed in 10 per cent H_2SO_4 for 5 minutes, washed again, and then plated for 5 to 10 seconds in a 1 per cent solution of platinic chloride in 0.3 per cent HCl. The plating current employed here is 0.025 ampere and 4.0 volts, giving a current density of 0.181 ampere per sq. cm. After being plated it is washed and then electrolyzed again in 10 per cent H_2SO_4 for 30 seconds. We have found that electrodes so prepared could be used for some time, if the electrodes were reelectrolyzed for 30 seconds in 10 per cent H_2SO_4 , before being used on a new solution. The great advantage of this electrode is that equilibrium is attained in 1 to 2 minutes.

heated, with constant stirring, for 15 minutes at 50°, after which it was squeezed to dryness through closely woven muslin. The dry residue was triturated with 200 to 500 cc. of cold water and again squeezed to dryness. This procedure, the heating and subsequent trituration, was repeated twice more. The resulting dry residue was then ground to a paste with an equal volume of powdered glass and with 2 cc. of $M/15$ K_2HPO_4 per gm. of meat. After standing for $\frac{1}{2}$ hour at room temperature the mass was centrifuged, the supernatant suspension (the enzyme solution) passed through cloth and set away preserved with toluene in a cold room at 2°. On several occasions when the first extract was found to be inactive, a second grinding with a solution of phosphate (1 cc. of $M/15$ K_2HPO_4 + 0.5 cc. of $M/15$ KH_2PO_4 per gm. of meat) and extraction yielded an active enzyme preparation.

The activity and the residual reduction of the enzyme suspensions so obtained were tested by the methods described by Lehmann. Enzyme preparations with reduction times greater than 50 minutes at 37° were discarded.

Preliminary experiments indicated that at 25° the reduction of the dye ceases before the attainment of equilibrium if the amount of enzyme employed is too small. After a number of trials the following reaction mixture was finally settled upon: enzyme solution, 2 cc.; phosphate buffer or water, 1.5 cc.; 0.0007 M methylene blue, or other dye solution, 0.5 cc.; 0.02 M succinate or fumarate, 0.5 cc. The pH determinations were made on duplicate mixtures except that water was substituted for the dye solution. It was found that the slight concentration of the solutions occurring during the evacuation left the pH of the mixtures, within the limits of accuracy of our measurements, unchanged.

Thionine and indigo tetrasulfonate were used in the extreme acid and alkaline solutions instead of methylene blue.

The succinic acid and fumaric acid employed were twice recrystallized from Eastman preparations. These gave colorless solutions with theoretical titration values. The melting points of the solids were respectively 188° and 281° (corrected).

In the experiments of Thunberg and of Lehmann, in most cases, the potential was determined only with equal concentrations of succinate and fumarate. In our routine procedure the potentials were measured simultaneously in three mixtures containing differ-

ent ratios of succinate to fumarate, as a rule 9:1, 5:5, and 1:9. In this way it was possible to detect with some assurance the presence of other interfering enzymes, such as fumarase, or other

TABLE I
Oxidation-Reduction Potentials of Succinic Acid-Fumaric Acid System at 25°

$\frac{\text{Succinate}}{\text{Fumarate}} = \frac{9}{1}$			$\frac{\text{Succinate}}{\text{Fumarate}} = \frac{5}{5}$			$\frac{\text{Succinate}}{\text{Fumarate}} = \frac{1}{9}$			Enzyme
pH	Phosphate concentration	-E	pH	Phosphate concentration	-E	pH	Phosphate concentration	-E	
	M			M			M		
6.19	0.2	0.440	6.10	0.2	0.440	6.10	0.2	0.435	Beef heart
6.67	0.05	0.438	6.25	0.2	0.437	6.19	0.2	0.437	
6.67	0.05	0.437	6.67	0.05	0.441	6.25	0.2	0.437	
6.76	0.2	0.444	6.67	0.05	0.438	6.67	0.05	0.439	
7.07	0.2	0.436	6.81	0.2	0.436	6.67	0.05	0.439	
7.08	0.2	0.436	7.07	0.2	0.434	6.76	0.2	0.434	
7.08	0.2	0.436	7.08	0.2	0.437	6.81	0.2	0.434	
7.08	0.2	0.437	7.08	0.2	0.435	7.07	0.2	0.434	
7.08	0.2	0.437	7.12	0.2	0.434	7.08	0.2	0.437	
7.08	0.2	0.440	7.47	0.2	0.437*	7.12	0.2	0.434*	
7.12	0.2	0.439				7.46	0.2	0.436	
7.47	0.2	0.439				7.47	0.2	0.440	
Mean.		0.438			0.437			0.436	
6.16	0.2	0.444	7.05	0.2	0.439	6.16	0.2	0.437	Beef diaphragm
7.05	0.2	0.439	7.12	0.03	0.435	7.05	0.2	0.437	
7.05	0.2	0.437	7.12	0.03	0.434	7.12	0.03	0.435	
7.12	0.03	0.433	7.90	0.2	0.436	7.90	0.2	0.434	
7.12	0.03	0.431							
Mean.		0.437			0.436			0.436	

* Values were obtained after the evacuated mixtures had been set away in a water bath for $\frac{1}{2}$ or 1 hour at 37°, until the methylene blue was decolorized and then set away at 25°.

oxidizing enzymes, by discrepancies between the values obtained with the different ratios and by the continuously negatively drifting potential, and in some of the earlier experiments such disturbing factors as air leaks through the agar-potassium chloride bridges.

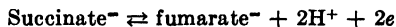
The values recorded in Table I are those of experiments in which a final potential was steady to within 0.1 millivolt for 1 hour or more. The validity of this selection of values we feel is demonstrated by the concordance among themselves of these steady values over a wide range of hydrogen ion activities, and metabolite ratios, by the correspondence of the mean value with that obtained by other workers at various temperatures and with various enzyme preparations, and finally by the coincidence of these experimental values with that calculated from the entropies, solubilities, ionization constants, and thermal data of succinic acid and fumaric acid.

The values set out in Table I show that the molal electrode potential, \tilde{E} , is independent (a) of the hydrogen ion activity in the range of pH 6.10 to 7.90, (b) of the source of the enzyme, (c) of the metabolite ratio employed, and (d) of the ionic strength of the solution.

The values marked with an asterisk were obtained after the evacuated mixtures had been set away in a water bath for $\frac{1}{2}$ hour or 1 hour at 37°, until the methylene blue was decolorized. The tubes were then removed to the air bath at 25°. In both tubes the color of the methylene blue was partially restored. This and the final values obtained confirm the reversibility of the reaction.

Theoretical Formulation

The term \tilde{E} in Table I corresponds to the molal electrode potential against the normal hydrogen electrode for the reaction



We have employed a somewhat different derivation of the electrode potential equation than that commonly employed, in order to obtain a clearer insight into the thermodynamic significance of the various terms. Though the mathematical relationships of the terms in the final equation, as Clark has emphasized (6), are independent of the mechanism postulated in its derivation, nevertheless the practice in the conventional derivation of assembling constants and including them in the characteristic potential obscures the thermodynamic significance of the various terms, because by including miscellaneous constants in the term E_0 , the postulated mechanism, *ipso facto*, is changed. Though no error is incurred

in such a change, since the chemical mechanism postulated is conventional only, and is chosen for its convenience, yet it is desirable for clarity to maintain throughout the derivation of an equation, and in its application to experimental results, when it is possible, the mechanism initially postulated, arbitrary or conventional though it be. Maintaining this consistency it is possible, in passing from system to system, to realize the significance of the various terms in the final equation, without the labor of rederiving these equations every time, which is necessary when a number of constants are assembled into a characteristic constant E_0 , whose meaning consequently may vary from system to system according to the constants included in it. We feel also that the fixing of attention on the free energy changes provides a "scaffolding" which conforms more closely to the features of the process than is obtained with the more conventional derivation of oxidation-reduction potential equations.³

The process of converting succinic acid to fumaric acid and hydrogen ions isothermally in any given solution may be considered as occurring in the following steps. 1 mol of the succinate ion is transferred from an infinitely large volume of solution where the total concentration of succinic acid in all its forms and the hydrogen ion activity are those of the experimental solution,

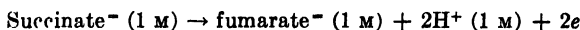
³ The following steps yield a general derivation of oxidation-reduction potential equations: (1) calculation of concentration of that form of reductant postulated in the mechanism obtaining at the experimental hydrogen ion activity and total concentration of reductant; (2) concentration of 1 mol of this form to 1 molal activity; (3) oxidation:—reductant (1 M) \rightarrow oxidant (1 M) + nH^+ (1 M) + ne ; (4) dilution of oxidant to the experimental activity and hydrogen ion activity (this step is equivalent to the sum of Steps 1 and 2); (5) dilution of n equivalents of hydrogen ions from 1 molal to experimental activity.

In this derivation the characteristic constant, \bar{E} , or E_0 corresponds to Step 3. The term $\frac{RT}{nF} \ln \frac{S_r}{S_0}$ is derived from Steps 2 to 4; the free energy change due to changing dissociation constants is obtained from the difference between the corresponding values calculated in Step 1 and Step 4. The term for the dependence on the hydrogen ion activity is derived from Step 5, and is always $\frac{nRT}{nF} \ln \frac{1}{(H^+)}$.

to another infinitely large volume of solution containing the succinate ion, fumarate ion, and hydrogen ion, all at molal activities. If S_i represents the total succinic acid in the initial solution, the molal free energy change in this step is

$$-\Delta F_1 = RT \ln \frac{K'_1 K'_2 \cdot S_i}{(H^+)^2 + K'_1(H^+) + K'_1 K'_2} \quad (1)$$

For the second step



we may designate the free energy change as $-\Delta\tilde{F}$.

The third step consists in the transfer of 1 mol of fumarate ion and of 2 mols of hydrogen ions from this hypothetical solution in which their activities are 1 molal to a solution in which the activities of the fumaric acid, and of the hydrogen ion, are those obtaining in the experimental solution. In this step the free energy change for the transfer of the fumarate ion is

$$-\Delta F_2 = RT \ln \frac{(H^+)^2 + K'_1(H^+) + K'_1 K'_2}{K'_1 K'_2 \cdot S_f} \quad (2)$$

and for the hydrogen ions

$$-\Delta F_3 = 2RT \ln \frac{1}{(H^+)} \quad (3)$$

The total free energy change therefore is,

$$-\Delta F_{\text{obs.}} = -\Delta F_1 - \Delta\tilde{F} - \Delta F_2 - \Delta F_3$$

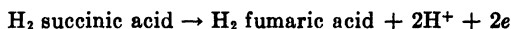
$$\begin{aligned} -\Delta F_{\text{obs.}} = & -\Delta\tilde{F} + RT \ln \frac{S_i}{S_f} + RT \ln \frac{K'_1 K'_2}{K'_1 K'_2} \times \\ & \frac{(H^+)^2 + K'_1(H^+) + K'_1 K'_2}{(H^+)^2 + K'_1(H^+) + K'_1 K'_2} + 2RT \ln \frac{1}{(H^+)} \end{aligned} \quad (4)$$

$$-\Delta F = EnF \quad (5)$$

$$\therefore E_{\text{obs.}} = \tilde{E} + \frac{RT}{nF} \ln \frac{S_i}{S_f} + 2 \frac{RT}{nF} \ln \frac{1}{(H^+)} + \frac{RT}{nF} \ln \frac{K'_1 K'_2}{K'_1 K'_2} \times$$

$$\frac{(H^+)^2 + K'_1(H^+) + K'_1K'_2}{(H^+)^2 + K_1(H^+) + K_1K_2} \quad (6)$$

The employment of an intermediate solution in which all the reactants are at 1 molal activity is open to the criticism that in a solution in which the hydrogen ion activity is 1 molal, the dissociation of both succinic acid and fumaric acid is completely suppressed. This criticism could have been avoided by employing the mechanism



Nevertheless we have preferred the mechanism with bivalent succinate and fumarate ions, because it corresponds more closely to the experimental conditions, and because the assumptions which must be made regarding differences in activity coefficients are less significant in the calculation of the free energy changes for the two dissociated forms than for the two undissociated forms. As Table I shows the potentials obtained show no systematic variation with the ionic strength. This we have taken as indicating that the ratio of the activity coefficients of succinate and fumarate remains constant, within the limits of accuracy of our measurements, over a range of ionic strengths varying from 0.09 to 0.6, and therefore is probably unity. The data given by Lehmann exhibit a similar independence of the potential and the ionic strength. On the other hand uncertainties regarding the values of the activity coefficients of succinic acid and fumaric acid would have introduced an uncertainty of 24 millivolts if the calculations had been based upon the mechanism involving the undissociated forms.

In the computation of the values of \tilde{E} in Table I the following data reported by Sihvonen (7) were employed for the variations of the titration constants with ionic strength.

Succinic Acid + KCl

$$\begin{aligned} pK'_{18^\circ} &= 4.213 - 0.998 \sqrt{\mu} + 1.27\mu \\ pK''_{18^\circ} &= 5.634 - 1.996 \sqrt{\mu} + 2.74\mu \\ pK'_{37^\circ} &= 4.182 - 1.030 \sqrt{\mu} + 1.34\mu \\ pK''_{37^\circ} &= 5.650 - 2.060 \sqrt{\mu} + 2.68\mu \end{aligned}$$

Fumaric Acid + KCl

$$pK'_{18^\circ} = 3.031 - 0.998 \sqrt{\mu} + 2.80\mu$$

$$pK''_{18^\circ} = 4.466 - 1.996 \sqrt{\mu} + 2.83\mu$$

$$pK'_{37^\circ} = 3.042 - 1.030 \sqrt{\mu} + 2.40\mu$$

$$pK''_{37^\circ} = 4.511 - 2.060 \sqrt{\mu} + 3.03\mu$$

The mean value obtained for the molal electrode potential at 25° for the succinate-enzyme-fumarate system is -0.437 volt, or $-20,140$ calories.

Employing the above titration data of Sihvonen and the equation of Cohn (8)

$$pH + \log \frac{KH_2PO_4}{K_2HPO_4} = 7.16 - \frac{1.5 \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}}$$

we have recalculated the data given by Lehmann. The corrections for the variations in the ionization of succinic acid and fumaric acid with ionic strength range from 0.0135 volt at pH 5.0 to 0.0002 volt at pH 7.0; and yield a good correspondence between calculated and observed potentials over this pH range. On the other hand the corrections employed by Lehmann, 0.0180 to 0.0002 volt respectively, give calculated values which are systematically more positive than the experimental values in the acid reactions.

In this connection it may be pointed out that the marked negative aberrations of the potentials from the calculated curve at hydrogen ion activities more alkaline than pH 7.56 observed by Lehmann, seem, from our observations, to be due to the interference of another enzymatic oxidation, which is not obtained with every enzyme preparation. As Table I shows we have succeeded at times in obtaining an enzyme preparation which did not show this effect even at pH 7.9. When this secondary reaction occurred there was a persistently negative drift of the potentials even after many hours and at the same time the values of different metabolite ratios were discordant. On the other hand when this effect was absent steady potentials were obtained with different metabolite ratios which were in good agreement with each other, and with the values obtained at other hydrogen ion activities.

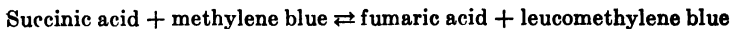
The data supplied by Lehmann, after the introduction of the corrections discussed above yield mean values for \bar{E} at 37° and at

18° of -0.430 and -0.443 volt respectively. These values are the same as those deduced by Lehmann from the potentials obtained in the neighborhood of pH 7.0 where the error due to the employment of faulty titration constants is negligible. Employing a linear temperature coefficient, the values of \bar{E} and $-\Delta F$ at 25° from the above are -0.438 volt and $-20,180$ calories, respectively.

The close correspondence between the values obtained by Lehmann at 37° and by the authors at 25° confirms not only the accuracy of the potentials obtained but also the value of the temperature coefficient. From this temperature coefficient by means of the Gibbs-Helmholtz equation Lehmann calculated ΔH to be $-29,850$ calories. The calculated value from purely thermal data given in Table V is $-29,800$ calories.

Two earlier potential measurements by Thunberg are quoted by Lehmann. The enzyme was prepared, presumably, from horse skeletal muscle, and contained fumarase. In order to prevent the hydrolysis of fumaric acid to *l*-malic acid 3 times its equivalent quantity of *l*-malic acid was added, the equilibrium ratio of *l*-malic acid to fumaric acid being 3. The first measurement was made colorimetrically at 30°, and at pH 6.7, and the result quoted is -0.015 volt. Since equivalent quantities of succinic and fumaric acids were used, the value of \bar{E} at 30° is -0.411 volt. A later presumably electrometric determination at pH 6.91 and 30° yielded a potential of -0.015 volt corresponding to $\bar{E} = -0.433$ volt. At 25° these become -0.415 and -0.436 volt respectively. These values correspond in calories to free energy changes of $-19,140$ and $-20,100$ respectively.

Quastel and Whetham with resting *Bacillus coli* at 45° obtained for the reaction



a value of 3.0 for the equilibrium constant. According to these authors the fumarase action under their conditions is very slow and their equilibrium value may be considered as unaffected by this secondary reaction. According to Clark, Cohen, and Gibbs (9) the potential of methylene blue at 30°, when the ratio of re-

ductant to oxidant is 1, is 0.004 volt. On the assumption that the temperature coefficient for methylene blue $\frac{dE_h}{dT} = -0.00135$, obtained by Clark, Cohen, and Gibbs at pH 8.62, obtains also at pH 7.2 and is linear up to 45°, the value for E'_0 at 45° at this pH would then be -0.016 volt.

Since at equilibrium the potential of the methylene blue system is the same as that of the succinate-fumarate system we may write $\frac{(\text{succinate})}{(\text{fumarate})} \times \frac{(\text{methylene blue})}{(\text{leucomethylene blue})} = 3$, when $\frac{(\text{methylene blue})}{(\text{leucomethylene blue})} = 1$, with a change in sign since here the reaction has been written reductant \rightarrow oxidant $+ 2e$.

$0.016 = \tilde{E} + \frac{RT}{nF} \ln 3 + \frac{RT}{F} 7.2$, the correction term for ionization being negligible at pH 7.2. Therefore $\tilde{E}_{318^\circ} = -0.424$.

With use of the same linear temperature coefficient $\tilde{E}_{298^\circ} = -0.438$ and $-\Delta F_{298^\circ} = -20,180$ calories.

Quastel and Whetham do not state the temperature at which their pH measurement was made, nor the concentration of the phosphate. We have assumed, therefore, in this calculation, that whatever error is incurred by taking the pH to have been 7.2 at 45° is neutralized by the error incurred by employing the potential for methylene blue at pH 7.2 instead of that which actually obtained at 45°.

The free energy change in the oxidation of succinic acid to fumaric acid was calculated from thermochemical data as follows:

Entropy of succinic acid, $S_{298^\circ} = 42.0$ (10); carbon, $S = 1.3$ (11); O_2 , $S = 49.03$ (12); H_2 , $S = 31.23$ (13).

Therefore the entropy of formation of solid succinic acid calculated from the reaction, $4C + 3H_2 + 2O_2 \rightarrow C_4H_6O_4$, is

$$\Delta S(s) = 42.0 - 4(1.3) - 3(31.23) - 2(49.03) = -155.0$$

The value of 31.23 for the entropy of molecular hydrogen employed here is considerably different from the older value given by Lewis and Randall of 29.44. This revised value for the entropy of hydrogen, due to Giaque (13), has been accepted by Parks and his collaborators and is now employed by them in their calcu-

lations of the entropies of organic compounds by means of the third law of thermodynamics from specific heat data.⁴

The heat of combustion of succinic acid at constant pressure was found by Verkade, Hartman, and Coops (14) at 19° to be 357,100 calories. Reduced to 25° and to its weight in vacuum the value becomes 356,900. With the value 94,240 calories obtained by Roth (15) for the heat of combustion of carbon to carbon dioxide and 68,310 calories for the heat of formation of water recently obtained by the United States Bureau of Standards, the heat of formation of solid succinic acid calculated from the reaction $C_4H_6O_4 + 3\frac{1}{2}O_2 \rightarrow 4CO_2 + 3H_2O$, is

$$\Delta H(s)_{298^\circ} = 356,900 - 4(94,240) - 3(68,310) = -225,000 \text{ calories}$$

Since $\Delta S = \frac{\Delta H - \Delta F}{T}$, the free energy of solid succinic acid therefore is,

$$\Delta F(s)_{298^\circ} = -225,000 + 298 \times 155 = -178,800 \text{ calories}$$

In order to estimate the free energy of solution of succinic acid the information necessary is the solubility and activity coefficient of the undissociated form of succinic acid in its saturated solution. From the International Critical Tables the mol fraction x of succinic acid in its saturated solution is given by the expression

$$-\log x = \frac{1}{T} \cdot (0.5223) (32,380) - 3.778 = 1.895 \text{ at } 25^\circ$$

For the calculation of the activity coefficient of undissociated succinic acid we have employed the data in the International Critical Tables on the lowering of the vapor pressure and of the freezing point of aqueous solutions; and in the evaluation of these data

⁴ Giaque states, "The value of the entropy of hydrogen which should be used in conjunction with data obtained from the third law of thermodynamics is 31.23 E.U. . . . It is obtained by subtracting the high temperature nuclear spin entropy $R \ln 4 = 2.75$ E.U. from the absolute entropy of hydrogen 33.98 - 2.75 = 31.23 E.U. This places hydrogen on the same basis as other molecules in most of which, and perhaps in all of which, the subtraction is taken care of by the fact that heat capacities are not usually measured below temperatures of a few degrees absolute."

the methods described by Lewis and Randall ((11) pp. 273, 286) involving the use of their h function with the vapor pressure lowering data, and their j function with the depression of the freezing point data. Table II contains the results of these computations showing the allowance made for the activity of the ions arising from the dissociation of succinic acid. No partial molal specific heat data for these solutions are available for computing the variation in the activities with temperature. We have, therefore, assumed that the activities of all the constituents of these

TABLE II

*Activities of Components of Succinic Acid in Aqueous Solutions from Vapor Pressure and Freezing Point Depression Data**

Molality	Total activity a_2	Activity of					Data employed
		H^+ a_+	Succi- nate= a_-	Undissociated form			
				Molality M	Activity a	Activity coeffi- cient	
M				M			
0.01	0.0100			0.0100	0.0100	1.00	Freezing point
0.05	0.0509			0.05	0.051	1.02	“ “
0.1	0.0988			0.1	0.099	0.99	“ “
0.2	0.1976			0.2	0.198	0.99	“ “
1.211	1.04	0.01	0.0092	1.19	1.02	0.86	Vapor pressure
2.817	2.36	0.0155	0.0139	2.78	2.33	0.84	“ “
4.021	3.22	0.0185	0.0163	3.98	3.19	0.80	“ “
4.722	3.59	0.0205	0.0176	4.68	3.55	0.76	“ “
8.030	6.02	0.0267	0.0225	7.97	5.97	0.75	“ “

* International critical tables of numerical data, physics, chemistry and technology, New York and London (1926).

solutions remain the same over the temperature range from approximately 0° to 100°, and have estimated the activity coefficient of the undissociated succinic acid in its saturated solution at 25° to be 0.87. It was estimated that in this solution 0.008 mol of succinic acid was dissociated. Since the molality of the saturated solution at 25° is 0.715, the activity of undissociated succinic acid is $0.87 \times 0.707 = 0.615$ molal. The free energy change, therefore, in the transfer from a saturated solution to one in which undissociated succinic acid is at 1 molal activity is

$$-\Delta F = RT \ln 0.615 = -288 \text{ calories}$$

The free energy of undissociated succinic acid in solution at 1 molal activity, therefore, is $-178,800 + 288 = -178,500$ calories.

For the reaction, H_2 succinic acid \rightarrow succinate $^-$ + $2H^+$, in which all the participants are at activities of 1 molal, the free energy change $-\Delta F = RT \ln K_1 K_2$, where K_1 and K_2 are defined by the equations,

$$K_1 = \frac{(H^+) (H \cdot \text{succinate}^-)}{(H_2 \text{ succinate})}, \text{ and } K_2 = \frac{(H^+) (\text{succinate}^-)}{(H \cdot \text{succinate}^-)}$$

From the data of Sihvonen, the calculated values of pK_1 and pK_2 at 25° and at infinite dilution, since we are dealing with activities and therefore with true equilibrium constants, are 4.201 and 5.641 respectively. The free energy change in ionization therefore is $-13,420$ calories. Hence the free energy of the bivalent succinate ion is $-178,510 + 13,420 = -165,090$ calories.

The entropy of solid fumaric acid at this temperature is 39.7 (10). The entropy of formation therefore is

$$\Delta S(s)_{298} = 39.7 - 4(1.3) - 2(31.23) - 2(49.03) = -126.0$$

For the heat of combustion of fumaric acid we have used Roth's value of 319,700 calories at 19° and weighed in air, which on a vacuum basis and at 25° becomes 319,300 calories (15). Parks and Huffman (10) have employed Stohmann's value which is 600 calories greater. In a private communication Professor Parks wrote that in their present revision of their data they "have rather arbitrarily given Roth's result twice the weight of Stohmann's and thus have taken the value 319,900 calories for 19° weighed in air. This gives 319,500 calories for 25° on a vacuum basis." We have preferred to ignore the earlier value and to give full weight to the value obtained by Roth. The difference between this value and the mean value employed by Parks is only 200 calories. The heat of formation of solid fumaric acid is, therefore,

$$\Delta H(s)_{298} = 319,300 - 4(94,240) - 2(68,310) = -194,280 \text{ calories}$$

The free energy of formation of solid fumaric acid is

$$\Delta F(s)_{298} = -194,280 + 298(126.0) = -156,720 \text{ calories}$$

A saturated solution of fumaric acid in water at 25° contains 0.061 gm. per 100 gm. of water (16). In its saturated solution the ionization is approximately 12.5 per cent, from which the molality of the undissociated form in the saturated solution is 0.0469. In the absence of data by which we might have estimated the activity coefficient of undissociated fumaric acid at this concentration, we have assumed that it is the same as that of succinic acid at this concentration, 1.0. The free energy change, therefore, in the transfer of 1 mol of undissociated fumaric acid from its saturated solution at 25° to one in which its activity is 1 molal is $RT \ln 0.053 = -1822$ calories.

The free energy, therefore, of undissociated fumaric acid in solution at 1 molal activity is

$$\Delta F(s)_{298^\circ} = -156,720 + 1820 = -154,900 \text{ calories}$$

The free energy change in ionization, $\text{H}_2 \text{ fumaric} \rightarrow \text{fumarate}^- + 2\text{H}^+$ is

$$RT \ln K_1 K_2 = -10,270 \text{ calories}$$

where K_1 and K_2 are the first and second hydrogen dissociation constants of fumaric acid.

The free energy of the bivalent fumarate ion at 1 molal activity therefore is

$$\Delta F(1 \text{ M}) = -154,980 + 10,270 = -144,630 \text{ calories}$$

Therefore in the reaction, $\text{succinate}^- (1 \text{ M}) \rightarrow \text{fumarate} (1 \text{ M}) + 2\text{H}^+ (1 \text{ M}) + 2e$.

$$- \Delta F_{298^\circ} = -165,090 + 144,630 = -20,460 \text{ calories}$$

This computation is summarized in Table III.

In Table IV are set out the free energy values for this reaction obtained from potential and equilibrium measurements and these are compared with the above calculated value. Excepting the first determination by Thunberg the correspondence is remarkable. The difference between the mean of the electrometric and equilibrium values and the thermal value is greater than the probable error in the estimation of activity coefficients, but is well within the experimental error of the direct heat measurements. For instance

an error in the entropy difference of 1 unit (Parks and Huffman state that an error of 2 units is possible) would amount to 300 calories, and would practically account for the whole difference between calculated and observed values. A considerably larger

TABLE III

Summary of the Calculation from Thermochemical Data of Free Energy Change in the Conversion of Succinic Acid to Fumaric Acid and Hydrogen Ions

	Succinic acid	Fumaric acid
	calories	calories
Free energy of formation of solid	-178,800	-156,720
" " " solution	+288	+1,820
" " " ionization	+13,420	+10,270
Standard free energy of bivalent ion	-165,090	-144,630
" " " change	-20,460	

TABLE IV

Comparison of Observed with Calculated Free Energy Changes in the Enzymatic Oxidation of Succinic Acid to Fumaric Acid

Investigator	Source of enzyme	Temperature of measurement	- ΔF	Difference from calculated value of - ΔF , - 20,460 calories (- $\Delta F_{obs.}$) - (- $\Delta F_{cal.}$)
		$^{\circ}\text{C.}$	calories	calories
Thunberg, 1925	Horse skeletal muscle	30	-19,140	1320
" 1928	" " "	30	-20,100	360
Lehmann	" " "	37	-20,180	280
		18		
Quastel and Whetham	Resting <i>Bacillus coli</i>	45	-20,180	280
Authors	Beef heart muscle	25	-20,140	320
"	" diaphragm	25	-20,140	320

error is also possible in the heat of combustion values as the discussion above of the heat of combustion of fumaric acid indicates. The employment of a fixed thermal value for the free energy change for purposes of comparison with the electrometric data shows the variations in the electrometric values. Had the mean electro-

metric value been employed as the fixed value for purposes of comparison, the variations in the calculated thermal values would have been greater than those shown in Table IV among the electrometric values.

It seems permissible, therefore, from the correspondence shown in Table IV, to conclude, as a first approximation, that the enzyme or enzymes which effect either *in vitro* or *in vivo* the oxidation of succinic acid to fumaric acid may be classed as "perfect catalysts."

The correspondence between the calculated and observed values for the change in heat content, ΔH , is also remarkably close. This is shown in Table V.

The close correspondence of the values for ΔH calculated from electrometric and from thermal data is additional confirmation

TABLE V
Heats of Formation of Succinate⁼ and Fumarate⁼

	Succinic acid	Fumaric acid
Heat of formation of undissociated solid.....	-225,000	-194,280
" " solution.....	+6,400	+5,900
" " ionization.....	-320	-740
" " formation of bivalent ion in solution...	-218,920	-189,120
$-\Delta H$ Succinate ⁼ \longrightarrow fumarate ⁼	-29,800	
$-\Delta H$ calculated from $\frac{dE}{dT}$	-29,850	

of the conclusion based upon the agreement between calculated and observed values for ΔF , that in this reaction the enzyme, regardless of the source of its preparation, or of its site of action, operates as a "perfect" catalyst, *i.e.* the reaction proceeds in a perfectly reversible manner, the heat and free energy changes being unaffected by the intervention of the catalyst.

The ratio, $\Delta F:\Delta H$, in this reaction is $20,200 : 29,850 = 0.68$. It was pointed out in a previous communication (17) that the ratio $\Delta F:\Delta H$ was nearly unity for the combustion of both tripalmitin and of glucose. Since the maximum amount of work derivable is practically equal to the value of $-\Delta F$, any difference in efficiency of fat and carbohydrate as fuels for muscular work must be ascribed to differences in intermediary metabolism. The

conversion of succinic acid to fumaric acid is an example in which the difference between ΔF and ΔH is quite large, and therefore the theoretical maximum work derivable from this reaction alone is much less than the total heat change. Since the formation of a double bond is a typical first stage in the oxidation of fatty acids it is probable that this considerable difference between ΔF and ΔH in the case of the oxidation of succinic acid to fumaric acid is typical of the oxidation of fatty acids in general. Of course the conversion of succinic acid to fumaric acid is only a "half reaction." Eventually an exothermic reduction of oxygen must occur, in which the ratio of $\Delta F:\Delta H$ is always nearer 1. Nevertheless even in the complete reaction involving oxygen, the chief responsibility for differences between ΔF and ΔH would rest with the oxidation of the organic metabolite. It must be added, of course, that ΔF will vary, also, with the actual concentrations of the metabolites and products of reaction, whereas ΔH will not be significantly changed.

SUMMARY

1. A modification of the Thunberg vacuum technique for the micro electrometric determination of oxidation-reduction potentials is described.

2. A general derivation of oxidation-reduction potential equations is presented possessing some advantages for purpose of thermodynamic calculations over the conventional derivation.

3. The molal electrode potential, \tilde{E} , for the succinate-enzyme-fumarate equilibrium was measured with enzymes prepared from beef heart, and from beef diaphragm, over a range of hydrogen ion activities from pH 6.15 to 7.9. It was found that the potential is independent (a) of the metabolite ratio employed, confirming the earlier observations of Lehmann, (b) of the hydrogen ion activity, and (c) of the ionic strength of the solution.

4. It is shown further that the value of -0.437 volt for the molal electrode potential, obtained with beef heart and with beef diaphragm, within the narrow limits of experimental error, is the same as that obtained with such different catalysts as resting *Bacillus coli*, or enzymes prepared from horse skeletal muscle.

5. The molal electrode potential of -0.437 volt corresponding to a standard free energy change at 25° for the reaction



of $-20,140$ calories, agrees very closely with the standard free energy change of $-20,460$ calories calculated from the entropies and other physicochemical properties of succinic acid and fumaric acid.

6. Similarly the heat of reaction, $-\Delta H$, $-29,850$ calories, calculated from the temperature coefficient of the potential is shown to be independent of the enzyme employed and agrees very closely with the value of $-29,800$ calories calculated from the thermal data.

7. This close correspondence between calculated and observed values of $-\Delta F$ and $-\Delta H$ is taken as proof that the enzyme promoting this reaction is a perfect catalyst; and that the enzyme probably operates in this manner *in vivo* as well as *in vitro*.

8. The correspondence between these calculated and observed values is additional confirmation of the third law of thermodynamics.

9. The difference between $-\Delta F$ and $-\Delta H$ in this reaction is discussed in relation to energy changes in intermediary metabolism.

10. The free energy of formation of the bivalent succinate ion at 1 molal activity at 25° was estimated at $-165,090$ calories, and the corresponding standard free energy of the bivalent fumarate ion at $-144,630$ calories.

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THE FREE ENERGY, HEAT, AND ENTROPY OF FORMATION OF *l*-MALIC ACID

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(Received for publication, May 11, 1931)

In the previous communication (1), it was shown that the free energy of the bivalent fumarate ion at 25° is -144,630 calories. In the present communication the measurement of the equilibrium at 25° between fumaric acid and malic acid in the presence of fumarase is reported, and from the values obtained computations are made of the free energy and heat of formation of the bivalent *l*-malate ion and of the free energy and entropy of solid *l*-malic acid.

It has been known for many years that a reversible hydrolysis of fumaric acid to *l*-malic acid occurs in the presence of minced muscle or of extracts made from it. The values obtained for the equilibrium constant have varied with different workers. Recently Lehmann (2) has described a potentiometric measurement of this equilibrium constant.

In order to obtain the value of ΔH for this reaction and thereby the means of computing the equilibrium constants at various temperatures, the authors carried out a number of such potential measurements at 25°.

In his employment of this potentiometric method the assumption was made by Lehmann that the only reactions taking place, even when the enzyme preparation contained fumarase, were: fumaric acid + $H_2O \rightarrow l$ -malic acid; and succinic acid + methylene blue \rightarrow fumaric acid + leucomethylene blue. The observations described below, of the authors, show that this is not generally true, and that the value of the equilibrium constant obtained from potentiometric measurements may not be accepted unreservedly. The measurements of Lehmann were made on either initially equi-

molar mixtures of succinic acid and fumaric acid, or on equimolar mixtures of these acids to which a quantity of *l*-malic acid was added corresponding to the estimated amount of *l*-malic acid which would be in equilibrium with the fumaric acid initially added.

The authors' measurements were made with initially 1:9, 5:5, and 9:1 ratios of succinic acid to fumaric acid, and at two different hydrogen ion concentrations. It was found that whereas with fumarase-free enzyme preparations the experimental value of n , *i.e.* the number of electrochemical equivalents involved in the electrode reaction, was 2, when fumarase was present the value of n was always greater than 2, varying from 2.17 to 2.26, giving a mean value of 2.22. Our interpretation of this fractional value for n is that some of the malic acid formed from the fumaric acid is oxidized and that the resulting leucomethylene blue reacts with some of the fumaric acid to form oxidized methylene blue and succinic acid. In support of this interpretation is the observation that in the presence of even fumarase-free enzyme preparations *l*-malic acid is oxidized with the reduction of methylene blue. In the absence of any other metabolite than malic acid complete reduction of the dye occurs. When fumaric acid is also added, the reduction of the methylene blue stops at a potential corresponding to a low ratio of succinic acid to fumaric acid. This cessation of the reaction suggests that when a small amount of the malic acid is oxidized, with the formation of a corresponding amount of succinic acid, an equilibrium is attained. That only a small amount of malic acid is oxidized is indicated also by the value of n being close to 2. These results show the danger of measuring reduction potentials with only one metabolite ratio. As Table I shows, the values for the equilibrium constant at the two different hydrogen ion concentrations for each metabolite ratio agree well with each other, yet the values with different metabolite ratios vary systematically.

In the absence of interfering secondary reactions the potential difference between the initially 9:1 and 1:9 mixtures of succinic acid to fumaric acid would have been 56 millivolts; the difference found experimentally was 51 millivolts. We have based our calculation of the value of the equilibrium constant on the potentials obtained with the initially 5:5 mixtures of succinic acid and fu-

maric acid. The possible error is approximately ± 2.5 millivolts. Though this uncertainty of 2.5 millivolts introduces a relatively large uncertainty into the value of the equilibrium constant, for the purpose of estimating the free energy of formation of *l*-malic acid from fumaric acid the error is quite small, amounting to not more than 150 calories.

The technique employed in these determinations was identical with that described in the previous communication (1), except that the enzyme preparation was modified so as to preserve the

TABLE I

Equilibrium Potentials of Succinic Acid-Fumaric Acid Mixtures in Presence of Fumarase-Containing Enzyme

Initial ratio of succinic acid to fumaric acid	pH	Potential, if no fumarase had been present E'_h	Potential observed E'_h	$-(E'_h - E''_h)$	$K \text{ for } \frac{(\text{malic})}{(\text{fumaric})} = K$
		volt	volt	volt	
9:1	6.81	-0.0054	+0.0104	0.0158	2.42
9:1	6.81	-0.0054	+0.0097	0.0151	2.24
9:1	7.12	+0.0128	+0.0280	0.0152	2.27
5:5	6.81	-0.0336	-0.0158	0.0178	2.99
5:5	6.81	-0.0336	-0.0156	0.0180	3.06
5:5	7.12	-0.0154	+0.0032	0.0186	3.25
5:5	7.12	-0.0154	+0.0027	0.0181	3.09
1:9	6.81	-0.0618	-0.0406	0.0212	4.20
1:9	6.81	-0.0618	-0.0415	0.0203	3.85
1:9	7.12	-0.0438	-0.0236	0.0200	3.74
1:9	7.12	-0.0438	-0.0231	0.0205	3.93

fumarase activity. Beef heart was finely minced, triturated, and washed five times with 0.25 per cent NaCl, once with distilled water, and then extracted, after grinding with powdered glass, as in the preparation of the fumarase-free enzyme suspension.

The results obtained are collected in Table I.

The equilibrium constant for the reaction



$$\frac{(\text{Malic})}{(\text{Fumaric})} = K$$

was calculated from the potentials (the secondary reaction being disregarded) as follows:

E'_A = potential observed when fumarase is absent from enzyme preparation

E''_A = potential observed in presence of fumarase

(Fum) = equilibrium concentration of fumaric acid in presence of fumarase

\therefore (Fum) (1 + K) = initial concentration of fumaric acid

$$\therefore E''_A = \tilde{E} + \frac{RT}{F} \text{pH} + \frac{RT}{nF} \ln \frac{(\text{Succ.})}{(\text{Fum})} + \text{correction for ionization}$$

$$\therefore E'_A - E''_A = \frac{RT}{nF} \ln \frac{\frac{(\text{Succ.})}{(\text{Fum}) (1 + K)}}{\frac{(\text{Succ.})}{(\text{Fum})}}$$

$$= \frac{RT}{nF} \ln \frac{1}{(1 + K)}$$

$$\therefore - (E'_A - E''_A) = \frac{RT}{nF} \ln (1 + K)$$

In the calculation of K in Table I we have taken the value of n as 2 in spite of the fact that the value calculated from the experimental results is 2.21. This value for n of 2.21 is based upon the assumption of a value of -0.437 volt for \tilde{E} which must be erroneous since it does not take into account the secondary reaction discussed above. A second erroneous assumption is made also in considering that the various ratios of succinic acid to fumaric acid maintain their initial relationships to each other after the attainment of equilibrium in the presence of the fumarase-containing preparation. This assumption would have been valid if, apart from the hydrolysis of the fumaric acid to *l*-malic acid, the reduction of the methylene blue had been the only significant reaction occurring here. On account of our inability to measure this secondary reaction it seemed preferable for the time being to accept only those values of the equilibrium constant calculated from the potentials of the initially 5:5 mixtures of succinic acid to fumaric

acid, and to take the value of n as 2. The mean of these values is 3.1. As Table I shows, these are intermediate between the diverging extremes: the final value of the ratio of succinic acid to fumaric acid is least divergent in this range from that calculated on the assumption that the fumaric acid is converted only to *l*-malic acid, and the rate of the change of the potentials with varying ratios of reductant to oxidant is least here. The maximum error in this approximation is probably much less than 2.5 millivolts, which corresponds to a change in the value of K of 0.75 and to differences in the free energy change of less than 150 calories.

Lehmann, at 37°, also calculating from the potentials of initially 5:5 ratios, and taking $n = 2$, obtained a mean value of 3.0. It seems probable that the enzyme preparations employed by Lehmann would have given divergences similar to those shown in Table I. Woolf measured this equilibrium with *Bacillus coli* as catalyst, in the presence of *l*-aspartate (3) at 37°. The equilibrium ratios of *l*-malate to fumarate were found to be 3.1 and 3.2. These values provide an independent check on the values for the equilibrium constant since the *l*-malic acid was measured polarimetrically.

The coincidence of the values for the equilibrium constant obtained with *Bacillus coli* and with enzyme preparations from minced horse and skeletal muscle shows, as in the case of the succinate-enzyme-fumarate equilibrium, that the equilibrium position is practically independent of the source of the enzyme.

In view of the uncertainty regarding the precise values of the equilibrium constants no reliance could be placed on a value of ΔH calculated from the equilibrium constants at 37° and 25°. Less error is likely to be incurred by assuming ΔH to be the same as ΔF , i.e. about 700 calories. The heat of formation of the bivalent *l*-malate ion may then be estimated from the more accurately determined values of the heats of formation of the bivalent fumarate ion and of water, which are respectively, -189,120 calories (1) and -68,310 calories, yielding for the heat of formation of bivalent *l*-malate ion a value of -258,100 calories.

Computation of the Free Energy and Entropy of Formation of l-Malic Acid

The fraction of the total malic acid in the bivalent form at pH 6.81 is 96.8 per cent and at pH 7.12, 97.8 per cent, when pK_1

= 3.48, and $\text{p}K_2 = 5.11$ (4) for the dissociation constants of *l*-malic acid. On account of the uncertainty of the value for the equilibrium constant we shall consider the malic acid as completely dissociated, and not introduce the trifling correction into the remaining fumaric acid for the undissociated malic acid. We shall write therefore



$$\Delta F_{\text{l-malate}} = -144,630 - 56,560 - 670 = -201,860 \text{ calories}$$

TABLE II

Activity Coefficients of Aqueous d-Tartaric Acid Solutions at High Concentrations

Molality	Activity coefficients from	
	Vapor pressure at 100°	Depression of freezing point
1.5		1.11
2.0	1.27	
3.0	1.49	1.24
4.0	1.73	1.35
5.0		1.48
5.3	2.06	
6.5	2.12	
10.0	2.74	

The free energy of ionization of *l*-malic acid

$$-\Delta F_{\text{ionization}} = RT \ln K_1 K_2 = -11,720 \text{ calories}$$

where K_1 and K_2 are respectively the first and second hydrogen ion dissociation constants. Therefore the free energy of formation of undissociated *l*-malic acid at 1 molal activity is

$$\Delta F_{(\text{l-malic}, 1 \text{ M})} = -201,860 - 11,720 = -212,580 \text{ calories}$$

The solubility of *l*-malic acid was found to be 100 gm. in 129 gm. of solution, corresponding to a mol fraction of 0.317. The mol fraction of molal malic acid in the undissociated state is $1:56.51 = 0.0177$. Since no data are available for the estimation of the activity coefficient of *l*-malic acid in its saturated solution, we have assumed that it is not very different in this respect from

d-tartaric acid for which vapor pressure and freezing point lowering data up to 10 and 5 molal solutions respectively are given in the International Critical Tables. By means of the *h* and *j* functions of Lewis and Randall (5) the activity coefficients of *d*-tartaric acid were computed from these data. These are set out in Table II.

It seems hardly probable that the discrepancies between the activity coefficients for *d*-tartaric acid calculated from vapor pressure and freezing point data can be due to experimental errors. More probably the differences are due to the large difference in temperature at which the two measurements were made. The accuracy of the measurements would not warrant taking into account the temperature coefficient of the partial molal specific heat content of *d*-tartaric acid, even if the data were available; especially as in any case an extrapolation from a 10 molal to a 22 molal solution (the molality of the saturated *l*-malic acid solution) is necessary. We have, therefore, taken the activity coefficient of *l*-malic acid in its saturated solution to be 2. We feel that this approximation does not incur an error in the estimation of the free energy of *l*-malic acid of more than 200 or 300 calories.

The ionization of malic acid in its saturated solution, which is negligible here, being disregarded the free energy of transfer at 25° from the solution in which its activity is molal to the saturated solution is $RT \ln \frac{0.0177}{0.317 \times 2} = -2120$ calories. The free energy of formation of solid undissociated malic acid at 25° is $-212,580 + 2120 = -210,460$ calories.

The heat of combustion of *l*-malic acid (solid) at 19° is 320,100 calories (6) which we may take as 320,000 calories at 25°. The heat of formation of *l*-malic acid in the reaction $C_4H_6O_5 + 3O_2 \rightarrow 4CO_2 + 3H_2O$ is therefore,

$$\Delta H = 320,000 - 4(94,240) - 3(68,310) = -261,890 \text{ calories}$$

From the relationship $\Delta S = \frac{\Delta H - \Delta F}{T}$, the entropy of formation of solid *l*-malic acid at 25°,

$$\Delta S = \frac{-261,890 + 210,460}{298} = -172.5 \text{ E.U.}$$

The entropy of solid *l*-malic acid since

$$S_{\text{malic}} - S_{(4\text{ C})} - S_{(3\text{ H}_2)} - S_{(2\text{ O}_2)} = -172.5 \text{ E.U.}$$

is

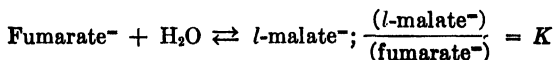
$$\begin{aligned} S(\text{solid})_{298^\circ} &= -172.5 + 4(1.3) + 3(31.23) + 2.5(49.03) \\ &= 49 \text{ E.U.} \end{aligned}$$

It is obvious that the value obtained for the free energy of *l*-malic acid cannot be considered as final. On the other hand the approximations cannot, it seems, have introduced a large error. The uncertainty regarding the equilibrium constants corresponds to 150 calories. The employment of titration constants at 18° and 25° instead of the dissociation constants at 25° and at infinite dilution, if we judge from the values for fumaric acid, does not amount to more than 100 calories, and the uncertainty regarding the activity coefficient of *l*-malic acid in its saturated solution to another 250 calories. Even if these errors were all in the same direction the total would be not more than 500 calories, which is negligible for most energy calculations in intermediary metabolism.

The entropy value depends also on the reliability of the heat of combustion. It is planned to obtain an independent determination of the entropy and free energy value of *l*-malic acid by specific heat measurements.

SUMMARY

1. The equilibrium constant for the reaction



was estimated from the electrometric measurements at 25° to be approximately 3.1.

2. One of the possible errors of the potentiometric method of measuring the equilibrium constant is demonstrated.

3. The value of the free energy of formation of *l*-malic acid (solid) at 25° was estimated at -210,450 calories, with an error not greater than ± 500 calories. The value of the free energy of bi-

valent *l*-malate ion at 1 molal activity was estimated at $-201,940 \pm 150$ calories; and the heat content at $-258,100$ calories.

4. The value of the entropy of solid *l*-malic acid at 25° was estimated at 49 E.U.

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THE ALLERGICALLY ACTIVE SUBSTANCE IN RAGWEED POLLEN. A CHEMICAL AND BIOLOGICAL STUDY*

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INTRODUCTION

The object of this work has been the isolation and identification of the fraction or fractions of pollen which were specifically active in clinical cases of hay fever. The fractions giving an immediate urticarial type of cutaneous reaction have been studied. No attempt was made to identify inactive material. The importance of a close relationship between chemical work and biological tests was evident.

In the preliminary report, two substances were described. Substance A, containing a large amount of carbohydrate, gave positive skin reactions in 90 per cent of a series of ragweed-sensitive cases and in 10 per cent it was inactive. Substance B, of protein nature and free from Substance A, gave reactions in all of the ragweed-sensitive cases tested.

Subsequent work here reported will show that Substance A, which could not be prepared free from nitrogen, was a mixture of small amounts of Substance B with carbohydrate material, since tests have shown that the active material in the so called Substance A was biologically identical with Substance B. Substance B has been isolated in a comparatively pure state and corresponds on chemical analysis and by its chemical reactions to a plant albumin.

* A preliminary report was read before the Society for the Study of Asthma and Allied Conditions, Sixth Annual Meeting, Atlantic City, May 3, 1930 (*J. Allergy*, 1, 470 (1930)).

HISTORICAL

Heyl (1), in a chemical analysis of ragweed pollen, isolated twenty-seven substances, but due to insufficient clinical tests with the products the results were disappointing in regard to allergic activity. Fatty acids, lecithin, resins, sucrose, glucose, nitrogenous bases, and phytosterols were isolated. A fraction composed of 75 per cent albumin and 25 per cent proteose had anaphylactogenic properties. Koessler (2) studied the nitrogen distribution in ragweed extracts and Gottlieb and Oppenheimer (3) prepared active material by alcoholic precipitation from saline extracts. Paton (4) reported the presence of eight enzymes in pollen. Bauman, Chudnoff, and Mackenzie (5) reported the presence of active material in a fraction that they considered globulin. Caulfield, Cohen, and Eadie (6) described an albumin-proteose fraction giving positive skin reactions. Coca and Grove (7) and Black and Moore (8) from a study of extracts concluded that the active substance of pollen was non-protein. Moore and Moore (9) have made a study of the nitrogen distribution in extracts of timothy, orchard grass, and ragweed pollen and Moore, Cromwell, and Moore (10) have described fractions of varying activity. Bernton, Jones, and Csonka (11) concluded from a preliminary study of protein fractions from timothy and ragweed pollen that the activity was not restricted to a single fraction.

EXPERIMENTAL

The pollen of the giant ragweed (*Ambrosia trifida*) has been used in this work except where otherwise specified. Preliminary work with timothy, low ragweed, and plantain pollen has indicated active material of a similar nature. Tests for activity have been made with solutions in bicarbonate-extracting fluid (12), buffered saline (13), or normal salt, that were in some measure comparable to the routine ragweed extracts. Whenever insoluble, the material was tested by the scratch method.

The direct method of testing extracts intradermally on sensitive individuals, while valuable for establishing activity, is not reliable for determining the amount of active substance. With the direct method a certain extract will often give an immediate wheal apparently identical with that produced by the same extract diluted 100 times.

In this study it was frequently necessary to determine the relative amounts of allergen by comparing certain fractions of the pollen with each other or with the routine extract of defatted pollen. Such comparisons may be quite accurately made by the indirect method (passive transfer of Prausnitz and Küstner (14)) as follows: An accurately measured amount (0.05 or 0.1 cc.) of serum

of ragweed-sensitive patients was injected into the skin of non-sensitive individuals. Three such sites were usually made in each arm. 2 or 3 days later measured amounts (0.05 or 0.1 cc.) of the extracts to be tested were injected into sensitized sites. Immediate reactions took place with the result that the sites were partially or completely desensitized. When any site was inactive to its own test extract it was then tested with the other solution which was being compared. Occasionally sites, frequently tested, may become refractory from tissue change or fatigue. In this event a negative reaction is not due to specific desensitization and may be

TABLE I
Comparison of Purified Albumin Extract 991 with Ragweed Extract, by Desensitization

Date of test..	Aug. 11			Aug. 13							
Sites*	0.1 cc. extract	N found per cc.	Result	0.05 cc. extract	N found per cc.	Result	0.05 cc. extract	N found per cc.	Result	Extract	Result
		mg.			mg.			mg.			
1, right	R	0.1	++++	R	0.1	—	991	0.1	—	Horse serum	++++
2, “										“ “	++++
3, left	991	0.1	++++	991	0.1	—	R	0.1	—	“ “	++++
4, “										“ “	++++
Control	R	0.1	—								
“	991	0.1	—								

* Injections in these sites were made on August 9.

misleading. To guard against this, sera were selected from cases sensitive to ragweed and one or more unrelated antigens, for example, horse serum. After ragweed tests were completed in any study and especially in cases where negative results were obtained, a test was made with horse serum and, if positive, refractoriness was ruled out. The negative response to ragweed extract was due to specific desensitization. In instances where sera used did not contain antibody to unrelated antigen the method was devised of supplying this by the addition of serum containing an antibody different from the one used for sensitizing sites for tests.

An example to be referred to later in the text is here cited (Table

1). Serum 30 was obtained from a patient suffering from asthma and hay fever and sensitive to ragweed and horse serum. On August 9, 0.1 cc. of this serum was injected intradermally into each of four sites, two on the right arm and two on the left arm of a non-sensitive individual. Extract 991 (purified albumin) was tested against a routine ragweed extract, Extract R, of defatted pollen.

This experiment, which is one of many, shows that the routine ragweed, Extract R, and the purified albumin of ragweed, Extract 991, were active in Sites 1 and 3 respectively. The second test with these substances in the same site 2 days later was negative; that is, the sites were desensitized. When Extract 991 was then put in Site 1 there was no response and when Extract R was put in Site 3 there was no reaction. This shows that the routine ragweed extract contained no active substance other than the albumin in Extract 991 for if another allergen were present a reaction would have resulted. Refractoriness was excluded by the subsequent reaction to horse serum. The desensitization was considered specific. This showed that the purified albumin contained the active substance present in the routine extract of ragweed pollen.

The extracts from whole pollen were made by removing the fatty material with ether or petroleum ether before aqueous extraction was carried out. This fatty oil when removed from dry pollen under anhydrous conditions was free from nitrogen and did not give immediate specific skin reactions. It was slightly water-soluble. In some cases on scratch test it produced a dermatitis as reported by Milford (15) and Bengtssen (16).

Sections of whole pollen grains stained with fat stains showed that this oily material was contained in the outer burr or shell of the pollen, acting more or less to inhibit water extraction.

1. Preliminary Work

A. Influence of Heat upon Activity of Pollen and Extracts

No allergically active volatile product could be isolated by a destructive distillation of whole ragweed pollen. A steam distillation gave a distillate with a pungent pollen odor, containing some ether-soluble fatty material with no skin activity. Activity remained in the distillation flask with the pollen residue.

The activity of dry whole ragweed pollen or petroleum ether-

defatted pollen was not measureably reduced by heating at 100-120° for 2 hours. An additional heating for 15 minutes at 200° resulted in browning the pollen with loss of activity.

Gay (17) made a study of the destructive action of heat under different conditions of dilution and pH concentration.

Tests, of passively sensitized sites, by the indirect method described on page 570 showed that heating Extract 901 for 1 hour in a boiling water bath had decidedly reduced its desensitizing capacity in comparison with the unheated preparation, Extract 901.

B. Chemical Analysis of Giant Ragweed Pollen

	<i>per cent</i>
Total volatile matter (102°).....	5.99
Vacuum drying loss.....	4.00
Ash.....	5.94
Nitrogen (dry whole pollen).....	4.11
“ (“ defatted pollen).....	4.63

Qualitative tests showed the presence of phosphorus but no sulfur. The ash was largely Ca and PO₄.

Material Removed by Extraction of Dry Whole Pollen with Anhydrous Solvents (Soxhlet)

	<i>per cent</i>
Petroleum ether, b.p. 30-50°.....	10.9
Benzene, b.p. 80°.....	11.8
Chloroform.....	15.2
Ethyl ether.....	11.3
Absolute alcohol.....	32.7

Petroleum ether, dry ethyl ether, and benzene extracts were found nitrogen-free by sodium fusion. Chloroform and 95 per cent alcohol extracts containing nitrogen and phosphorus showed faint activity with some loss of activity in the residues. A 95 per cent alcohol extract of dry pollen residue following water extraction was inactive.

In all subsequent work petroleum ether-defatted ragweed pollen was used.

Material Removed by Successive Extraction

3 gm. of ragweed pollen were extracted at room temperature with successive 100 cc. volumes of absolute alcohol, 95 per cent alcohol,

and 70 per cent alcohol and water. Nitrogen was determined on each extract as well as on the dry residue and the amount of material removed in alcoholic extracts was calculated from the weight of the dry residue. On evaporation of 10 cc. of the clear water extract to dryness at 100°, 0.0093 gm. of material resulted. This material contained 17 per cent ash, mainly calcium phosphate, and 8.6 per cent nitrogen calculated from the nitrogen in 10 cc. of the extract. The results are given in Table II.

The water extract contained the bulk of activity and therefore its nitrogen included the active nitrogen. Since 3 gm. of pollen in 100 cc. of water gave extracts of about 0.3 mg. of nitrogen per cc., but one-third of the water-extracted nitrogen was related to

TABLE II
Material Removed from Ragweed Pollen by Successive Extraction

	Material extracted	N found per cc.	N removed	Activity
	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>	
Absolute alcohol.....	12.2	0.14	0.45	—
95 per cent “.....	8.5	0.10	0.36	—
70 “ “.....	14.1	0.18	0.58	+
Water.....	3.0	0.08	0.25	++++
Residue.....			3.32	—
Total.....	37.8	0.50	4.96*	

* The defatted pollen contained 4.63 per cent N.

the activity. The 70 per cent alcohol contained some active material. Subsequent work showed the importance of these data.

Determination of pH of Pollen Extracts

A colorimetric determination of the pH of pollen extracts showed that distilled water extracts of ragweed and timothy pollen had a pH of 5.3 to 5.5. Bicarbonate-extracting fluid extracts had a pH of 7.6. A water extract of ragweed pollen which had been first extracted with absolute alcohol had a pH of 6.2. The absolute alcohol extract after evaporation and after being taken up in water had a pH of 4.8.

It seemed probable that the bicarbonate-extracting fluid favored the stability of routine extracts by giving slightly alkaline solu-

tions. Since the isoelectric point of most plant proteins (18) lies in the range of pH 4 to 5, the most favorable range for their precipitation, slight alkalinity favors the stability of these colloidal solutions.

C. Nitrogen Content and Activity of Water and Bicarbonate Extracts

18 hour water extracts of ragweed pollen prepared from 3 gm. of pollen per 100 cc. contained 0.3 mg. of nitrogen per cc., while a similar bicarbonate extract contained 0.4 mg. of nitrogen per cc. Skin tests showed no detectable difference in activity; hence the active material was as soluble in water as in alkaline medium.

TABLE III

Water Extracts of Ragweed Pollen and Pollen after Additional Cold Absolute and 95 Per Cent Alcohol Extraction

Prepared from 3 gm. per 100 cc.

	Pollen	Pollen after absolute and 95 per cent C ₂ H ₅ OH extraction
Material removed by water, <i>per cent.</i>	29.6	21.6
N per cc., <i>mg.</i>	0.32	0.26
Free reducing substances as glucose, <i>per cent.</i> ...	1.0	0.0
Total reducing substances after hydrolysis, <i>per cent.</i>	5.2	5.5
Activity.....	++++	++++

A 3 hour bicarbonate extraction of ragweed pollen was as complete as a 6, 18, or 72 hour extraction, showing the ready solubility of active material. Extracts of long duration were objectionable due to yielding turbid extracts giving precipitation on standing.

No difference in activity could be determined by direct skin tests in extracts made with different amounts of pollen in the same volume of extractive when dilutions were made on a nitrogen or weight basis.

A study of twelve successive water extracts of ragweed pollen showed that a 3 hour water extraction removed practically all the extractable nitrogen and all the reducing substances with the exception of a small constant factor as well as most of the active material.

D. Reducing Substances in Pollen and Pollen Extracts

Active extracts of ragweed pollen reduced Benedict's solution, showing free reducing substances with heavier reduction after acid hydrolysis. The reducing substances before hydrolysis were determined by clearing the extracts with mercuric nitrate solution, neutralizing with sodium bicarbonate, removing the last traces of mercury with hydrogen sulfide, and determining the free reducing substances by the gravimetric method of Allihn. Total reducing substances were determined similarly after hydrolyzing the extracts with 10 per cent sulfuric acid in a boiling water bath for 5 hours. Reducing substances are reported either as weight of Cu_2O per volume or as percentage glucose.

The ragweed pollen contained 9.5 per cent reducing substances after hydrolysis. The pollen residue after water extraction contained 4.5 per cent reducing substances.

Cold absolute and 95 per cent alcohol extracts of the ragweed pollen removed 16 per cent of the pollen. These extracts contained only free reducing substances and some water-soluble nitrogen and were inactive (see Table III).

The water extract following the alcohol extraction described above was active and contained no free reducing substances.

In addition to the nitrogen content of the active water extracts the presence of material of a glucosidic nature, giving reducing substances after hydrolysis, indicated that such material might be responsible for activity.

E. Comparison of Nitrogen Content and Reducing Substances in Distilled Water Extracts of Ragweed and Cattail Pollen

Since extensive clinical tests by one of the authors (Cooke) had failed to show sensitivity to the wind-borne cattail pollen (*Typha angustifolia*), it was of interest to compare it with an allergically active pollen such as ragweed (Table IV).

Cattail pollen extracts were free from the froth characteristic of ragweed extracts. Reducing substances were present entirely in the free state in cattail extract since reduction after hydrolysis was less, indicating some destruction during hydrolysis. In ragweed extracts a larger amount of these reducing substances resulted after hydrolysis, indicating material of the nature of a glucoside and the absence of such material in cattail further suggested this material

might be responsible for activity in ragweed. Furthermore, the cattail pollen extract on the addition of alcohol to 90 per cent concentration gave none of the typical gum precipitated from ragweed extract. This gum gave reduction only after hydrolysis.

Cattail pollen extracts did not precipitate at 50 per cent alcoholic concentration. As will be shown later, the active substance of ragweed pollen extract was precipitated at this point. This probably explains the clinical inactivity of cattail pollen.

F. Precipitation of Active Material from Pollen Extracts by Alcohol or Acetone

Preliminary experiments with distilled water extracts of ragweed, timothy, and plantain pollen showed that when alcohol or acetone was added to produce a final concentration of 90 per cent, the active

TABLE IV
Comparison of Cattail Pollen with Ragweed Pollen

	Ragweed extract, 3 gm. per 100 cc.	Cattail extract, 3 gm. per 100 cc.
Material removed by water, <i>per cent</i>	29.6	26.7
N per cc., <i>mg</i>	0.32	0.29
Free reducing substances as glucose, <i>per cent</i>	1.0	7.2
Total reducing substances after hydrolysis, <i>per cent</i>	5.2	5.3

substance was practically all in the precipitate. The clear, alcoholic or acetone supernatant liquid, after being evaporated and taken up in saline, showed very slight activity by direct skin test. From a water extract, one precipitation by alcohol to 90 per cent concentration gave a precipitate amounting to 7 per cent. This precipitate was not completely soluble in water. The soluble portion was taken up in water and reprecipitated by alcohol at 90 per cent concentration. By repeating this process a water-soluble, active material resulted which contained a high percentage of reducing substances on hydrolysis and was designated Substance A.

In order to have active material for study, 47 gm. of pollen were treated with cold absolute, then 95 per cent alcohol, as described on page 576, in order to remove inert water-soluble nitrogenous material and free reducing substances. The pollen was then

extracted for 4 hours with 350 cc. of distilled water at a temperature of 7°. The clear filtrate at a volume of 350 cc. was treated with 350 cc. of alcohol and within 3 hours a flocculent precipitate settled which when removed by the centrifuge left a clear solution. This material, Precipitate 761, when dry equalled 1.3 per cent of the pollen. To the clear alcoholic solution, alcohol was added to make the final volume 3200 cc. Precipitate 762 settled out after standing for 18 hours and it was collected by the centrifuge, washed with 95 per cent alcohol, dried, and equalled 4.7 per cent of the pollen.

Precipitate 761 was separated into a water-insoluble material, Precipitate 761-a, active on scratch test, which was shown later to be mainly coagulated Substance B and a small amount of carbohydrate material similar to Precipitate 762, Substance A. Precipitate 762 was dissolved in water, filtered, and the solution divided. One portion was precipitated by adding alcohol to 90 per cent, giving Precipitate 762-a; the other portion was similarly precipitated with acetone, giving Precipitate 762-b.

Ragweed pollen was washed in the cold with absolute and 95 per cent alcohol. The pollen residue was extracted with water and the clear filtrate was precipitated by adding an equal amount of alcohol. This precipitate was removed and the clear filtrate was further precipitated by adding alcohol to a final concentration of 90 per cent. This second precipitate was dissolved in water, filtered, and precipitated by the addition of alcohol to 90 per cent concentration for six times, giving Precipitate 856, Substance A (see Table VI).

Since Substance A, as prepared above, was soluble in 70 per cent alcohol, pollen after being washed with cold absolute and 95 per cent alcohol was extracted with 70 per cent alcohol. This filtered extract was precipitated by adding alcohol to 90 per cent concentration. This precipitate was taken up alternately in 70 per cent alcohol and water, and precipitated by the addition of alcohol to 90 per cent concentration for six times giving Precipitate 876.

Solutions containing from 0.2 to 0.3 gm. per 100 cc. of Precipitate 762, 856, or 876 (Substance A) contained nitrogen. The reducing substances after hydrolysis were about equal to routine ragweed extracts. These solutions of precipitates were active in direct skin tests in 90 per cent of a series of sensitive cases. However, 10 per cent of the cases gave reactions to routine ragweed

TABLE V
Comparison by Desensitization of Substance A, Solution of Precipitate 856, and of Substance B, Extract 883
 0.05 cc. of the solution was used in each test and 0.05 cc. of serum was injected into each site.

Date of test...	Nov. 27			Nov. 29				Dec. 1			
	Solution used	N found per cc.	Result	Solution used	N found per cc.	Result	Solution used	Solution used	N found per cc.	Result	Result
1, right	856	0.01	++	856	0.01	—	883		mg.		
2, "	856	0.1	+++	856	0.1	—	883				
3, "				883	0.1	+++					
4, left	883	0.01	+++	883	0.1	+++		883	0.1	—	—
5, "	883	0.1	+++	883	0.1	—	856				
6, "	883	0.01	+++	883	0.01	—		856	0.1	—	+++

* Injections in these sites were made on November 25.

pollen extracts, but negative reactions to solutions of these precipitates. This at first indicated that there might be two active substances in pollen, one of them this gummy glucosidic material, Substance A.

Attempts to prepare Precipitate 856 or 876 free from nitrogen were unsuccessful. The observation was made that material as first precipitated from the water extract with alcohol was more active in producing sneezing in a sensitive individual than the purified preparations, Precipitate 856 or 876.

In order to determine whether this glucoside-nitrogen-containing fraction (Substance A) was a specific allergen and distinct from the non-glucoside fraction containing nitrogen (Substance B),

TABLE VI
Analysis of Precipitates

Precipitate*	Nitrogen	Phosphorus	Glucose (reducing substances)	Ash†
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
762	4.38	2.58	48.8	
762-a	3.95	3.02	48.8	
762-b	6.53	2.76	48.8	
856	4.46	1.42	57.0	4.9
876	5.70	0.38	60.8	2.4

* All the precipitates on test showed activity.

† The ash contained Ca and PO₄.

desensitization tests were made. The following is a typical example. Results in all cases were identical.

M. G., aged 20 years, not sensitive to ragweed extract, volunteered for the experiment. Three sites were made in each arm with 0.05 cc. of serum in each site. The serum was obtained from a patient sensitive, clinically and by test, to ragweed pollen. The tests were made with solutions of Precipitate 856, the glucoside fraction, and Extract 883, described in detail later, which was free from glucoside. Solutions in each case were made on a nitrogen basis expressed in mg. per cc., 0.05 cc. being used in each test injection. Control tests with a solution of Precipitate 856 and Extract 883 were negative (Table V).

Tests in Sites 1 and 2 showed that Precipitate 856 desensitized sites against its own solution but not against Extract 883 in equiva-

lent solutions. In Sites 5 and 6, Extract 883 desensitized against itself and against Precipitate 856. It is particularly important to note in Site 6 that two injections of Extract 883 (0.01 mg. per cc. N) desensitized against desensitization caused by Precipitate 856 (0.1 mg. per cc. N) but the site was still reactive to Extract 883 (0.1 mg. per cc. N).

The conclusion must be that Precipitate 856 contained a small amount of the same active substance as Extract 883. Since Extract 883 contained no glucoside, this type of substance did not play a part as antigen in ragweed pollen extracts. Our former assumption of two antigens in this pollen was incorrect. There is no active glucoside (Substance A).

The nitrogen contents of Precipitates 856 and 876 (Table VI) were sufficient to explain their activity as due to small amounts of protein material. Further evidence for this conclusion was seen in the reduction of activity on repeated precipitation by alcohol, probably due to denaturation. Complete precipitation of pollen extracts and solutions of Precipitate 856 or 876 with phosphotungstic acid removed all the activity again, indicating the protein nature of the activity. Solutions of Precipitates 856 and 876 gave negative protein reactions, such as biuret, xanthoproteic, and Millon's. The high percentage of carbohydrates obscured these tests, but they gave a Molisch reaction and precipitation with basic lead acetate, phosphotungstic acid, and tannic acid. These solutions formed a froth on shaking, gave a reddish-brown color with concentrated sulfuric acid, and gave reduction only after hydrolysis. Hydrolyzed solutions gave tests for pentoses with aniline acetate paper and phloroglucine. With phenylhydrazine, a typical orange-yellow osazone melting at 160° resulted, indicating arabinose. These solutions were levorotatory before hydrolysis, showing no rotation afterwards, indicating protein material. In general, vegetable proteins are levorotatory. Purified Substance B was levorotatory; which was further evidence of its presence in Substance A.

2. Isolation and Identification of the Pollen Allergen

A. Separation of Substance A from Substance B

Since 70 per cent alcohol removed most of Substance A from the absolute and 95 per cent alcohol-washed pollen, different

strengths of alcohol were tried to see if one could be found which would remove Substance A without removing any great quantity of the active Substance B.

1.5 gm. of the absolute and 95 per cent alcohol-washed ragweed pollen were extracted for 18 hours with 50 cc. of 85 per cent alcohol, Extract 901-a. This extract was collected and the pollen washed with an additional 100 cc. of the same strength alcohol to remove the last traces of soluble material. The pollen residue was dried, weighed, and then extracted with 50 cc. of distilled water (Extract 901). The extracts were analyzed for nitrogen and reducing substances on hydrolysis. The water extract was tested for activity after saline dilution, and the alcoholic extract after evaporation was tested by the scratch test. The procedure was repeated with

TABLE VII
Extractions at Different Alcohol Concentrations

	Extract 901-a (85 per cent alcohol)	Extract 901 (H ₂ O)*	Extract 902-a (80 per cent alcohol)	Extract 902 (H ₂ O)	Extract 903-a (70 per cent alcohol)	Extract 903 (H ₂ O)
Material extracted, per cent..	19.4	11.5	21.8	8.5	24.9	5.9
N per cc., mg.....	0.22	0.14	0.25	0.12	0.35	0.09
Cu ₂ O per 10 cc., gm.....	0.0116	0.0170	0.0142	0.0132	0.021	0.005
Activity.....	+	++++	++	+++	++	+++

* Each alcohol extraction was followed by a water extraction as stated in the text.

use of 80 per cent and 70 per cent alcohol instead of 85 per cent alcohol (Table VII).

In concentrations lower than 95 per cent, alcohol removed the active material, but 70 per cent alcohol removed practically all the water-soluble gum, Substance A, since water extracts following this treatment gave no higher reducing substances after hydrolysis than a constant factor given by successive inactive extracts of pollen residues (see page 575). The water extract, Extract 903, was active in all the cases tested.

50 gm. of absolute and 95 per cent alcohol-washed pollen were treated with six changes of 70 per cent alcohol in portions of 125 cc., with frequent shaking. A 5 gm. sample of the pollen was removed and further washed with 70 per cent alcohol to remove

the last traces of soluble material. 3 gm. of this were extracted with 100 cc. of bicarbonate fluid giving Extract 871, Substance B.

The remainder of the pollen was extracted with 100 cc. of water and the clear filtrate precipitated with an equal amount of alcohol, giving a flocculent precipitate, Precipitate 873 of 0.3 per cent. This precipitate was removed and the clear solution was precipitated by addition of alcohol to 90 per cent concentration. The resulting material, Precipitate 874 of 1.3 per cent, was similar in character to Precipitate 876 and was Substance A not completely removed by the 70 per cent alcohol from such a large amount of pollen.

In order to have an extract of ragweed pollen completely free from Substance A for desensitization tests (see page 580) a sample

TABLE VIII
Analysis of Extracts of Pollen Residues

3 gm. per 100 cc. of saline were used.

Pollen residues	N per cc.	Cu ₂ O in 10 cc.	Substance	Activity
	<i>mg.</i>	<i>gm.</i>		
Pollen after absolute and 95 per cent alcohol extraction....	0.26	0.0353	A + B	++++
Pollen Extract 871 after 70 per cent alcohol extraction.....	0.11	0.0164	B + A	+++
Pollen Extract 883 after 50 per cent alcohol extraction.....	0.11	0.0090	B	+++

of the absolute and 95 per cent alcohol-washed pollen after exhaustive extraction with 70 per cent alcohol was finally extracted with two 100 cc. volumes of 50 per cent alcohol. The dry pollen residue was extracted with 3 gm. to 100 cc. of physiologic salt, and gave Extract 883, which analysis showed was free from Substance A (Table VIII).

Extract 883, Substance B, in desensitization tests, completely desensitized sites so that routine ragweed extracts gave no reaction. This showed that Substance B contained the biologically active material of the ragweed pollen, indicating there was only one active material in ragweed extracts.

Solutions Made from 0.2 Gm. of Precipitates in 100 Cc. Water

Precipitate No.	Pollen	Cu ₂ O per 10 cc.	Substance	Activity
	<i>per cent</i>	<i>gm.</i>		
873	0.3	0.0083	B	+++
874	1.3	0.0190	A + B	+
876	4.6	0.0268	A + B	+

Precipitate 873 resulting at 50 per cent alcoholic concentration from a water extract of 70 per cent alcohol-washed pollen was active in all cases tested. This was Substance B. Precipitates 874 and 876, inactive in about 10 per cent of the cases tested, were undoubtedly carbohydrate material containing small amounts of Substance B.

B. Isolation and Identification of Substance B

Substance B, Extract 883, gave positive biuret reaction and precipitation with Millon's reagent, phosphotungstic acid, and tannic acid. It contained nitrogen and its solubility in distilled water indicated protein material of albuminous nature.

45 gm. of ragweed pollen were washed with absolute and 95 per cent alcohol and finally with about 600 cc. of 85 per cent alcohol until most of the brownish gum had been removed. This facilitated the purification of the small amount of protein. The dry pollen residue was extracted with 150 cc. of water. The filtered water extract was treated with 150 cc. of saturated ammonium sulfate solution. After standing overnight no precipitate resulted and globulins were considered to be absent. The solution was saturated with ammonium sulfate and an albuminous substance separated, floating on the surface. The precipitate was dissolved in 100 cc. of water and 60 gm. of ammonium sulfate were added, when a permanent turbidity resulted. The pH was adjusted to 4.6 with 0.2 N sulfuric acid. An albuminous material, yellow in color, separated and after standing for 4 days it had a granular appearance and settled to the bottom of the flask. This precipitate was dissolved in water and similarly precipitated five times. The ammonium sulfate filtrate was practically inactive. The final precipitate was a very faint yellow in color and typically albuminous in nature. After being dissolved in water and dialyzed free from sulfate in a hardened membrane, the final solution, Extract

973, after Seitz filtration, measured 50 cc. It contained 0.02 mg. of nitrogen per cc., gave a positive biuret test, but negative Molisch test, showing the absence of carbohydrate material. On evaporation 10 cc. gave 0.0025 gm. of material.

The biological tests for comparing this preparation with routine ragweed extracts were carried out according to the method shown on page 570. Extract 973 desensitized passive sites completely so that subsequent tests with routine extracts were negative. This proved to be the case in all experiments done and showed that this preparation contained all of the active substances of ragweed pollen.

A 1:1000 dilution of Extract 973 containing 0.0000002 gm. of material per cc. and 0.00000002 gm. of nitrogen per cc. gave a positive reaction in a moderately sensitive case. Therefore we do not feel that the failure to demonstrate differences in nitrogen content by a Kjeldahl determination on active extracts after tryptic digestion (Coca and Grove (7) and Black and Moore (8)) was sufficient evidence on which to conclude that protein was absent and was not the active material.

Active material was precipitated by saturation with magnesium sulfate, but precipitation was not complete as subsequent saturation with ammonium sulfate resulted in further precipitation of active material.

188 gm. of ragweed pollen were washed with absolute and 95 per cent alcohol and finally 85 per cent alcohol as described. The dried residue was extracted for 5 hours with distilled water and filtered. The filtrate and washings, measuring 600 cc., were saturated with ammonium sulfate, and the precipitate filtered off. A second water extraction of the pollen residue on saturation with ammonium sulfate gave only a small amount of precipitate, showing that one extraction with water removed most of the active material. The two precipitates were combined, washed well with saturated ammonium sulfate solution, dissolved in water, and dialyzed in a hardened membrane against distilled water until free from sulfate. The solution was filtered and precipitated five times by about three-fourths saturation with ammonium sulfate and the pH was adjusted to 4.6. After the last precipitation, the precipitate was practically colorless. It was dissolved in water, dialyzed free from sulfate, and after Seitz filtration measured 200 cc. This clear solu-

tion, Extract 991, gave a froth on shaking, contained 0.2 mg. of nitrogen per cc., gave a positive biuret reaction, a precipitate with picric acid and with Millon's reagent, but a negative Molisch test. It was levorotatory. On evaporation at 100° a coagulated albuminous material precipitated, amounting to 0.0009 gm. per cc. It contained no phosphorus and 10 cc. of the solution after hydrolysis and clearing with mercuric nitrate gave no reduction. With this preparation it was impossible to demonstrate a precipitin test with ragweed-sensitive serum. The desensitization tests with this preparation are shown on page 571. By similar tests it was shown to be identical with Extract 973.

The remainder of the solution, about 160 cc., was precipitated with ammonium sulfate at pH 4.6. The precipitate was dissolved in 15 cc. of water and dialyzed free from SO_4 against distilled water. The final volume, 30 cc., was evaporated to 0.5 volume in a vacuum desiccator, when it was treated with 190 cc. of absolute alcohol. A white flocculent precipitate separated which was removed, dried at 100°, and submitted to analysis. Evaporation of the alcohol showed complete precipitation. The dried material produced violent sneezing in a slightly sensitive case.

Analysis of material dried at 100°: N 12.59, C 47.90, H 6.09, residue 4.90.

Percentage calculated after correcting for the 4.90 per cent residue: N 13.52, C 50.37, H 6.40.

The residue was composed of a small amount of Ca and SO_4 . Since the material was precipitated from a solution containing sulfuric acid, a small amount would be held by the protein and not removed completely by dialysis. No phosphorus was present.

The typical elementary analysis, high nitrogen content, as well as a negative Molisch test, no reduction after hydrolysis, and the absence of phosphorus showed the material was a simple protein. The material was characteristic of albumin since it was soluble in distilled water, precipitated by saturation with ammonium sulfate, coagulated on boiling with resulting lowering of activity and solubility, and gave protein tests.

Although very active in high dilutions and giving direct skin tests as great or greater than routine extracts, when dilutions were made on a nitrogen basis, the purified material probably became

somewhat less active during the process of isolation. In desensitization tests the purified material, on a nitrogen basis, desensitized sites against routine extracts, on a nitrogen basis. The purified material was not as strong as might have been expected since about two-thirds of the nitrogen in routine ragweed extracts has been shown to be inactive.

The elementary analyses on the small amounts of material obtainable were made possible by the use of the microchemical methods of Pregl through the courtesy of Professor Niederl, Mr. Silbert, and Mr. Saschek of New York University, to whom we wish to express our thanks and appreciation.

SUMMARY

1. The direct skin test while valuable for establishing activity was not satisfactory for determining the relative amount of active substance in various solutions. Such accurate determinations were made possible by quantitative desensitization of passively sensitized sites.

2. Cold absolute and 95 per cent alcohol removed water-soluble reducing substances, nitrogenous material, and acidity, but no active substance. 70 per cent alcohol removed a large amount of inert gum and some active substance leaving the bulk of active substance in the pollen residue.

3. From the pollen of the giant ragweed the active substance that produces the symptoms and skin reactions in individuals clinically sensitive to ragweed pollen (hay fever) has been isolated. This substance by chemical reactions and elementary analysis was typical of a simple protein of albuminous nature. Desensitization experiments have shown that this was the only active substance.

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THE DETERMINATION OF HEMOGLOBIN IN MINUTE AMOUNTS OF BLOOD BY WU'S METHOD*

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Despite the multiplicity of methods for the determination of the hemoglobin concentration of the blood, there appears to be no procedure available that requires less than 10 to 20 c.mm. While such volumes are usually considered quite small it may at times be difficult to secure even these amounts by skin puncture, without at least being tempted to squeeze the surrounding tissues in an effort to bring more blood to the surface. Any manipulation that involves "milking" or otherwise forcefully expressing the blood may cause surprisingly large errors in the hemoglobin determination and, contrary to what might be supposed, the errors usually lead to high rather than low values. Thus, an anemic rat, bled by snipping the end of the tail, may through faulty technique show apparently normal values. We have found that the volume of the smallest drop of blood that can be conveniently obtained even from the tail of a new born mouse, weighing less than 1.5 gm., is about 0.5 c.mm. From slightly larger animals 1.0 c.mm. can be easily obtained. On these minute samples of blood the hemoglobin concentration can be accurately determined by means of the method described herein. Because of the small volume required, it enables one to exercise the best technique in the collection of the sample, such as wiping off the first drop that appears after skin puncture and filling the pipette with freely flowing blood.

* A preliminary account of these experiments was presented at the Meetings of the American Society of Biological Chemists in Montreal, Canada, April 11, 1931.

The data in this paper are taken from a dissertation to be presented by Reginald W. Baker in partial fulfillment of the requirements for the degree of Master of Arts, Western Reserve University, 1931.

The method is essentially the pseudoperoxidase reaction described by Wu (1) in 1923. Wu developed the well known benzdine test for blood into a quantitative procedure and showed that the reaction was due entirely to hemoglobin. The method does not appear to have been used much and, indeed, we were unable to get check results by following the original directions. A thorough study was therefore made of the factors affecting the color development and as a result an improved technique was developed that we feel should render Wu's unique method as useful to others as it has been in some of our studies. It is necessary to adhere to the following directions exactly, as a perusal of the paper of Clark, Cohen, and Gibbs (2) on the oxidation of benzidine will show that many factors affect the color formation, and all of these must be standardized in order to obtain accurate results.

Solutions Required

Benzidine Reagent—Dissolve 2 gm. of a good quality benzidine in 20 cc. of glacial acetic acid with the aid of gentle heat. Allow the mixture to cool and dilute it to 100 cc. with distilled water. Decolorize by shaking with 0.5 to 1.0 gm. of decolorizing carbon and allow it to stand with occasional stirring for about 15 minutes. Filter and store in a brown bottle. If the solution becomes dark it may again be treated with carbon. For this reagent it is essential to use a good grade of benzidine. To ascertain whether a sample is suitable, prepare the reagent as described, place 2 cc. in a test-tube, and add 1 cc. of 3.0 per cent H_2O_2 . No color should develop in 1 hour. While poor samples of benzidine may be improved by being dissolved in twice their weight of warm 95 per cent alcohol, by addition of charcoal and refluxing for 15 minutes, pouring into 5 volumes of recently boiled and cooled (below 60°) distilled water, filtering, and drying in the vacuum oven, it is better to purchase a good brand at the beginning.

20 Per Cent Acetic Acid—This is made simply by diluting glacial acetic acid with 4 volumes of water.

Hydrogen Peroxide Solution—The ordinary 3 per cent solution is titrated with potassium permanganate (U. S. P.)¹ to make certain that it is the proper strength. For use, this is freshly

¹ United States Pharmacopœia X, Philadelphia, 217 (1926).

diluted with 4 volumes of water to give a 0.6 per cent solution. The strength may vary from 0.5 to 0.75 per cent H_2O_2 .

Standard Blood Solution—A sample of about 10 cc. of oxalated or defibrinated blood is analyzed for its oxygen capacity, according to the method of Van Slyke and Neill (3). The figure for oxygen capacity multiplied by the factor 0.746 is called hemoglobin. The blood is diluted with 1 per cent boric acid to give a solution having 20 mg. of hemoglobin per cc. A total volume of 25 cc. of solution is more than enough to last 1 month. For use, the standard blood is shaken and 0.5 cc. diluted to 200 cc. with distilled water. This dilute standard must be prepared fresh daily from the stock solution, and the latter should be kept at about 5° . The use of boric acid as a blood preservative was introduced by Brown and Hill (4).

Procedure

A blood sample of suitable volume is secured from a free flowing source and diluted by blowing into 2000 volumes of water. The pipette should be filled several times with the water and emptied, according to the usual technique. For human subjects, one may use a blood counting pipette if it has been previously calibrated to the 0.5 mark, this volume being about 0.005 cc. The pipette may be calibrated by weighing the amount of mercury delivered or, more conveniently, by comparison of the amount of blood delivered with an amount delivered by an accurately calibrated 1.0 cc. pipette.

If the volume of blood taken for analysis is 0.005 cc., the sample is blown into 10 cc. of water. If the volume is 0.001 cc., the blood may be blown into 2 cc. of water, or even 3 cc., in the latter case giving a dilution of 1:3000 but allowing duplicate determinations to be made upon the one sample. In every case 1 cc. of the thoroughly mixed and diluted blood is used for the determination. This is added to 2 cc. of the benzidine reagent, that has been previously carefully measured into a test-tube graduated at 25 cc. 1 cc. of 0.6 per cent H_2O_2 is then added. The solution turns blue in color, gradually increases in intensity, and becomes purple. The reaction should be permitted to proceed for at least 1 hour, at the end of which time the mixture is diluted to the mark with 20 per cent acetic acid, stoppered with a paraffined cork, inverted several times to mix, and, after 8 minutes, read in a colorimeter

against a standard set at 10.0 mm. The standard is prepared in exactly the same way, 1 cc. of dilute blood solution containing 0.05 mg. of hemoglobin being used. The calculations are very simple.

$$\text{Concentration in gm. per 100 cc. blood} = \frac{S}{R} \times \text{amount of Hb in standard} \times \frac{100}{\text{volume of sample in cc.}} \times \frac{\text{total volume of diluted blood sample}}{1 \text{ cc.}}$$

where S = reading of standard and R = reading of unknown. With a standard containing 0.05 mg. of Hb, set at 10.0 mm., and a blood sample of 0.005 cc. diluted to 10.0 cc., the equation reduces to: concentration in gm. per 100 cc. = $\frac{100}{R}$.

DISCUSSION

Order of Addition of Reagents—Wu laid considerable stress upon the necessity of adding the blood to the benzidine and the H_2O_2 to the mixture of the benzidine and blood, because low values were obtained when, instead, the benzidine was added to the blood. Probably a greater error, we find, occurs when H_2O_2 comes in contact with dilute blood unmixed with benzidine. For example, 5 cc. of 3 per cent H_2O_2 were mixed with 5 cc. of blood diluted 2000 times, and 1 cc. immediately pipetted into 2 cc. of the benzidine reagent in another tube. The time required for the mixing and the transference could not have been more than about 30 seconds, but no color developed even when more H_2O_2 was added. The substitution of 0.6 per cent H_2O_2 does not entirely obviate this danger, for after about 30 seconds contact the color was found to be two-thirds of the normal value, and after 3 minutes contact no color developed. The fact that dilute H_2O_2 destroys the pseudoperoxidase activity of dilute blood solutions indicates that under no circumstances should one allow drops of dilute blood to cling to the sides of the tube above the benzidine reagent where they run the danger of being "washed down" by the peroxide.

Effect of Concentrations of Reacting Substances on Time and Intensity of Color Development—The color produced is roughly proportional to the volume and concentration of the benzidine reagent.

Therefore, this solution must be measured accurately. Also, in order to have the hemoglobin concentration as the single variable, the volume of the reaction mixture should be kept constant by using the same amounts of benzidine, diluted blood, and H_2O_2 solutions for all determinations.

Wu used a 3 per cent H_2O_2 solution for the reaction, and allowed the unknowns and standard tubes to stand 15 minutes before dilution. If the digestions were allowed to proceed longer than 30 minutes the benzidine was destroyed by the H_2O_2 and higher values were obtained. The necessity of diluting the tubes at a particular time is very inconvenient when many determinations are to be made. Our procedure involves the substitution of a much more dilute H_2O_2 solution and necessitates a longer period of standing. We were led to this change quite by accident because a particular bottle labelled 3 per cent H_2O_2 was found to work very satisfactorily and gave perfect checks when eight to twelve tubes were made up with the same amount of blood in each. It was later found that the actual strength of the particular H_2O_2 solution was only 0.7 per cent by permanganate titration. Accordingly, the effect of concentration of the H_2O_2 was investigated. It was found that concentrations of 0.5, 0.6, and 0.75 per cent gave similar good results, but that 0.25 per cent required longer to develop the full color, while 1.0 and 2.0 per cent solutions had no apparent advantage. The smoothness of the reaction when 0.6 per cent H_2O_2 is used is indicated by the curve in Fig. 1. This was constructed by plotting the apparent hemoglobin concentration against time. It is to be seen that the color increases gradually until at 1 hour the maximum has been attained. No change was noted even in tubes allowed to stand 3 hours. Thus, there is no destruction of the benzidine with 0.6 per cent H_2O_2 at room temperature within this time.

The greater accuracy that we have obtained with the use of 0.6 per cent H_2O_2 overcomes the disadvantage of requiring a longer time for the reaction. The fact that no attention is required to avoid letting the reaction go too long is also an advantage. We feel these better results are due in part to a lessened tendency for the destruction of the hemoglobin activity by stronger peroxide solutions and in part to the avoidance of the direct oxidation of the benzidine. Incidentally, the use of dilute H_2O_2 eliminates the

occasional turbidity due to the precipitation of the traces of H_2SO_4 that are to be found as a preservative in some samples of 3 per cent peroxide.

Under the conditions of the reaction the hemoglobin concentration of the diluted blood is the only variable factor. To ascertain the relationship between hemoglobin concentration and the color

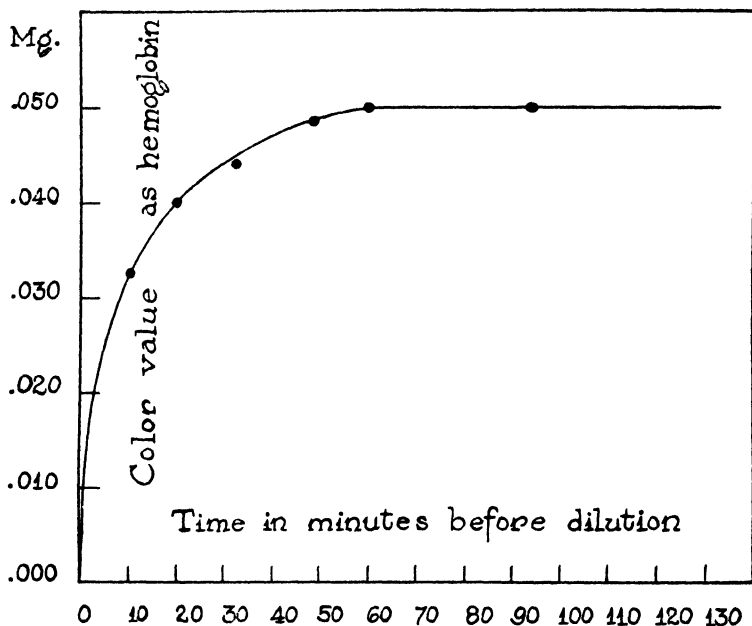


FIG. 1. The increase in color value in relation to time of reaction is shown. Each sample contained 2 cc. of benzidine reagent, 0.05 mg. of hemoglobin, and 1 cc. of 0.6 per cent H_2O_2 . The temperature was that of the room, 23° . While not shown on the chart, no change in color was observed even when the reaction was allowed to proceed 3 hours.

development a series of proportionality tests was made by diluting one sample of blood to different volumes with water and using 1 cc. of each dilution for analysis. The results are shown in Fig. 2. It is to be noted that only one sample, that containing 0.05 mg. of hemoglobin, was used as the standard. Theoretical proportionality was obtained from 0.01 mg. to 0.10 mg. of hemoglobin. Below the lower limit the colors are too dilute to match conven-

iently unless a weaker standard is used, and above the 0.10 mg. limit the results tend to be low. This range represents concentrations of hemoglobin varying from 2.0 to 20.0 gm. per 100 cc., if the blood is diluted 2000 times. It covers the extremely low figures that may be found in severe experimental anemias, and the

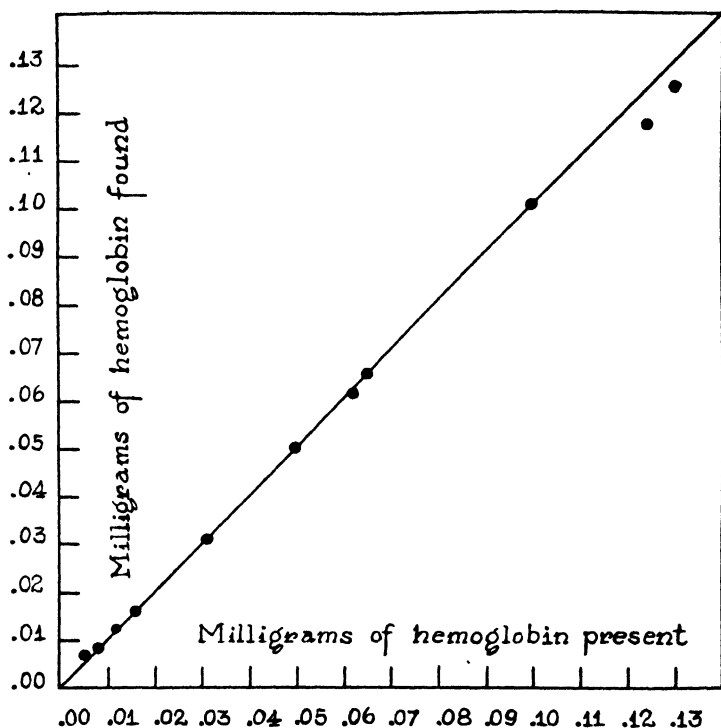


FIG. 2. The diagonal line represents true proportionality between hemoglobin present and color produced. The black circles represent actual observations, and show that with a single standard containing 0.05 mg. of hemoglobin the range of accuracy is from 0.01 mg. to 0.10 mg. of hemoglobin. Above this range the results tend to be low, and below 0.01 mg. the color is too dilute to match conveniently.

high values obtained in polycythemia, blood concentration, and early infancy. For hemoglobin determinations outside this range the dilution may be altered or standards selected that more nearly match the color of the unknown.

Other Blood Pigments—Wu proved that oxyhemoglobin, hemoglobin, methemoglobin, and carboxyhemoglobin all gave color values equivalent to hemoglobin. Our results have completely confirmed this, and also the peculiar observation that acid hematin gives only about 50 to 80 per cent of the color of an equivalent quantity of hemoglobin. If a large excess of potassium ferricyanide is present in a methemoglobin preparation it will itself affect the reaction.

Temperature—Increase of temperature does not speed up the time required for attainment of maximum color value. Tubes

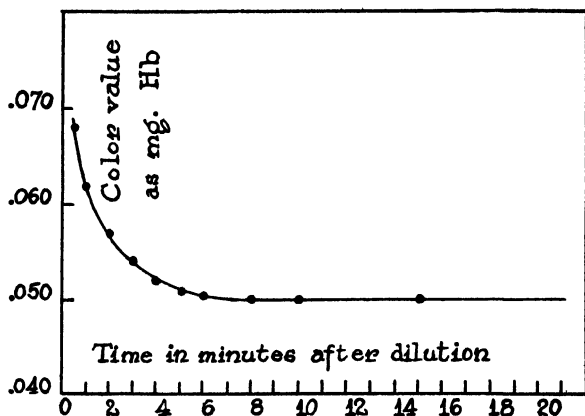


FIG. 3. The diminution in color after dilution of the benzidine-blood-peroxide mixture is shown. After 8 minutes the color is constant and remains so for at least 24 hours.

kept in a bath at 40° match exactly those made up at 20°, if both have been allowed to stand for 1 hour. A temperature of 60° gives an apparent increase in color without changing the slope of the time curve. This seems to be due to a destruction of the benzidine, because such solutions develop a brownish and somewhat turbid color.

Hydrogen Ion Concentration—The hydrogen ion concentration of the mixture affects the nature and the depth of color. The reaction mixture is ordinarily purple and has a pH of about 3.0. Addition of small amounts of strong alkali to the solution tends to give an increased color, addition of strong acid changes the color to a

brown, which is also much weaker. When the usual blood-benzidine- H_2O_2 mixture is diluted to 25 cc. with 20 per cent acetic acid a crystal clear wine-red solution is produced, having a pH of about 2.3. If the dilution is made with water or a glycoll-HCl buffer of pH 3.0, a purple solution is obtained, and on standing a purple precipitate slowly settles out. Dilution with glacial acetic acid yields a clear brown solution. Dilution with 0.1 N HCl also produces a brown color, and dilution with 0.005 N HCl gives a red solution with a faint cloudiness. The acetate ion, therefore, exerts an influence on the solubility of the colored products of the reaction.

The color change on dilution with 20 per cent acetic acid is not instantaneous. Observation has shown that 3 minutes are re-

TABLE I

Comparison of Colorimetric Hemoglobin Estimations with Values Calculated from Oxygen Capacity

Source of sample	Standard blood	Hb in gm. per 100 cc.	
		O_2 capacity $\times 0.746$	Present method
Mouse	Human I	12.4	12.7
Rooster	" I	13.7	13.2
Man	" II	9.3	9.0
Rabbit	" II	12.2	12.0
Man	" II	5.5	5.2
"	" II	11.5	11.5
"	Rabbit I	12.5	12.6
"	Human III	12.9	12.8

quired for all trace of the purple hue to disappear. The color value continues to diminish, as shown in Fig. 3, but reaches an equilibrium in 8 minutes. Thereafter, the color remains constant for at least 24 hours. There is no such change in intensity if the dilution is made with water or a glycoll-HCl buffer at pH 3.0, indicating that the diminution in color is associated with the change of hydrogen ion concentration. However, other factors are also involved for, with some samples of benzidine we have recently tried, a dark red instead of a purple color is obtained in the reaction mixture, and no alteration in color value occurs after dilution. This factor we are studying further. Because of the great extent of the color change usually obtained after dilution, it seems advisable

either to test for this phenomenon with each sample of benzidine, or to allow at least 8 minutes before reading in the colorimeter. There appeared to be no material advantage from attempting to control the acidity further by the introduction of a buffer solution.

Comparison with Oxygen Capacity Method—The present colorimetric method was checked against the values obtained by multiplying the oxygen capacity figures, determined according to the technique of Van Slyke and Neill, by 0.746. The blood samples were oxalated specimens from normal persons, hospital patients, and different species of animals. The results are presented in Table I and show a very good correspondence of the two methods.

TABLE II

Comparison of Hemoglobin Concentrations in Peripheral and Systemic Bloods of Suckling and Weanling Mice

Mouse No.	Body weight <i>gm.</i>	Hb in gm. per 100 cc.	
		Tail sample 0.00091 cc.	Carotid sample 0.0079 cc.
1	7.1	7.6	7.4
2	9.3	7.4	7.9
3	15.5	8.7	8.7
4	11.5	8.7	8.9
5	9.7	9.7	9.7
6	9.4	9.9	10.1
7	11.0	10.6	10.7
8	11.7	11.2	11.2
9	8.2	11.2	11.8

For these colorimetric estimations 1.0 cc. of blood was diluted to 2 liters and duplicate determinations made upon the diluted sample. Incidentally, these determinations also showed that each sample of blood when preserved with boric acid kept unaltered for 1 month. Probably, standard solutions could be kept for longer periods, but we have made no attempt to use any solution beyond 1 month.

Accuracy of Hemoglobin Estimations on Minute Samples of Blood—Preliminary experiments satisfied us that 1.0 c.mm. of blood could be accurately measured, blown into 2.0 cc. of water, and the hemoglobin content measured upon 1 cc. of the laked blood. In these trials, special micro pipettes were used. We wish to express

our appreciation of the help of Dr. A. T. Shohl of Western Reserve University in making these small pipettes. To demonstrate the accuracy of measuring minute volumes of blood, samples that happened to be 0.00091 cc. were taken from the tails of small mice and the hemoglobin concentration determined. The mice were 14 and 21 days old, the body weights ranging from 7 to 15 gm., depending upon age and the number of young permitted in the litter, and all the animals showed varying degrees of nutritional anemia. The mice were then etherized, an incision made through the neck muscles, and the carotid artery and jugular vein cut. A large drop of blood was collected on a watch-glass, and 0.0079 cc. immediately taken and blown into 20 cc. of water. Determinations of the hemoglobin concentration were made in duplicate upon this sample and compared with the values obtained with the tail sample. The results of these measurements are presented in Table II. They show that tail samples, when obtained by allowing the blood to flow freely, have the same concentration as systemic blood. This has been demonstrated by McCay (5) with rats. Because it has been shown that the supposed differences between systemic and peripheral blood do not ordinarily exist in rats and mice, one is inclined to ascribe also to faulty technique at least some of the discrepancies that have been reputed to occur in normal persons.

Applications—The method described herein has been found particularly valuable for determining the hemoglobin concentration of rats suffering from anemia brought about by an exclusive milk diet. Accurate determinations can be made upon small volumes of blood even when the concentration of hemoglobin is below 3 gm. per 100 cc. Moreover, if desired, samples can be taken daily with a minimal loss of blood to the animal. The method permits the estimation of the hemoglobin concentration in small animals such as baby rats and mice without sacrificing them. It has also been applied to the estimation of low hemoglobin concentrations in human subjects. In cases having as little as 5 gm. of hemoglobin per 100 cc. an easily obtained sample of 0.005 cc. of blood from the finger suffices for duplicate determinations.

CONCLUSIONS AND SUMMARY

Wu's method for estimating hemoglobin in blood by means of benzidine and H_2O_2 has been studied, and an improved technique

described. Sources of error in the application of the method have been pointed out. The method has been checked by parallel determinations with the oxygen capacity procedure of Van Slyke and Neill, normal and pathological human blood being used, and blood from several species of animals. By means of the present method, estimations of the hemoglobin concentration can be made upon samples of blood that measure as little as 0.001 cc. The maximum error should not be more than ± 4 per cent.

The authors desire to express their indebtedness to the continued advice and criticisms of Professor Victor C. Myers during the progress of these experiments.

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INFLUENCE OF SOLVENTS ON THE ACTIVATION OF ERGOSTEROL*

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In previous communications (1, 2) we described a parallel series of biologic and spectrographic measurements made on alcoholic solutions of ergosterol after different periods of irradiation. It was shown that the quantity of vitamin D in the photochemical reaction product rapidly rose to a maximum and gradually declined to zero, the parallel changes in absorption spectra indicating merely a transition from ergosterol to an isoergosterol-like substance, with nothing attributable to the vitamin.

As to the spectrographic changes, apart from vitamin formation, there is evidence that they are determined by such factors as the duration of exposure (1, 3-7), the wave-lengths of the ultra-violet light (7, 8), the amount of oxygen present (1, 2, 4, 5, 7), and lastly the nature of the solvent. We have emphasized that the spectrographic changes are no measure of antiricketic potency (1). To gain knowledge of the rise and decline of potency, it is usually necessary to prepare an activation curve, but because of the large number of animals required, this important interpretive aid in studies on activation has suffered neglect. It is strange that investigators who are careful with physical measurements remain content with bioassays of a lower order of accuracy.

Theoretically, it should be possible to activate ergosterol in any indifferent solvent which is sufficiently transparent to pass at least a portion of the wave-lengths which ergosterol absorbs.

* Excerpts from a paper, "Studies on the Activation of Ergosterol," presented at The Thirteenth International Physiological Congress, Boston, August 20, 1929.

That the solvent need not be freely transparent is shown by the fact that, when dissolved in arachis oil and exposed in thin layers, ergosterol can be activated to attain a cod liver oil coefficient of about 200,000.

The number of solvents that are transparent to the short wavelength side of the ergosterol absorption curve is limited, but alcohol, cyclohexane, and ether may be taken as examples. Each of these, when pure, transmits a high percentage of radiations between 230 $m\mu$ and the visible region, and hence might be expected to give similar results under similar conditions of exposure.

In the present experiment we investigated the influence of solvents by means of activation curves, using cyclohexane and ether in comparison with alcohol. The technique of irradiation was as previously described (1). Briefly restated, purified ergosterol ($[\alpha]_D^{20} = -132^\circ$ in CHCl_3) was dissolved in the desired solvent, 1 gm. per liter. The solutions were exposed in a 2 cm. homogeneous quartz cell touching the window of a Kromayer (water-cooled) mercury arc, for different time intervals. The bioassays were performed and interpreted in accordance with the precision technique described in our "Critique of the Line Test" (9). At least 100 rats were used for each activation curve, so that the probable error for the curves was only ± 4 per cent.

The activation curves for cyclohexane and ether are shown in Fig. 1, together with the original curve for alcohol. Although these curves possess the same general shape, the quantitative differences which they reveal are remarkable. With alcohol, the maximum cod liver oil coefficient was reached in the shortest time, 22.5 minutes, but the maximum was the lowest (250,000), and the decline in potency was the most rapid. After 3 hours and 18 minutes all antiricketic activity ceased. With cyclohexane, the maximum was reached in 27 minutes, but the maximum was somewhat higher (330,000). The decline was much more gradual, the reaction product showing a cod liver oil coefficient of 25,000 even after 14 hours of exposure. With ether the maximum was reached in 4 hours, 12 minutes, but the maximum was by far the highest (710,000). The decline in potency was relatively more rapid than with cyclohexane, yet even after 18 hours of exposure the reaction product showed a cod liver oil coefficient of 25,000. The curve for activation in ether is particularly strik-

ing in comparison with that in alcohol, for in ether the attainment of maximum potency required an hour more of exposure than was required in alcohol for the entire cycle of rise and decline.

A series of spectrographic examinations was made in parallel with the bioassays on the ether and cyclohexane solutions. The absorption spectra resembled those previously obtained with alcoholic solutions, except that the isoergosterol band was never so prominent. In these, and in many other spectra that we have

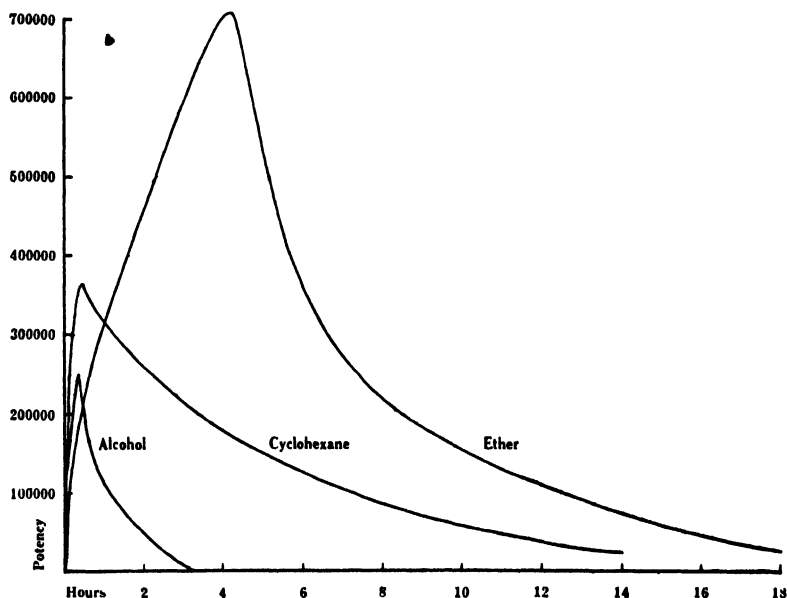


FIG. 1. Activation curves of ergosterol irradiated in alcohol, cyclohexane, and ether.

had occasion to make, there is no structure definitely attributable to the vitamin. They depict, apparently, a mixture of decomposition, or by-products, the make-up of which is influenced rather strongly by the conditions of irradiation.

We have no satisfactory explanation for the observed influence of solvents on activation. Mercury lamps are known to fail with prolonged use, but our results were not due to this cause. The activating power of the lamp was tested at intervals by irra-

diating alcoholic solutions. Quantitative assays revealed only a slight loss of efficiency, which was taken into account in plotting the activation curves.

One might presume that the different solvents had different protective, antioxidative actions. The quartz cell was always closely filled and tightly corked, but aside from this, no extraordinary precautions to exclude traces of oxygen were taken. While it is known that traces of oxygen affect the absorption spectra, especially during the early stages of exposure, it is a fact that comparatively large amounts do not preclude good activation. Tentatively it will suffice to view our results as a specific solvent effect.

SUMMARY

Activation curves of ergosterol irradiated in alcohol, cyclohexane, and ether had the same general shape, but widely different dimensions. Of these three solvents, ether permitted by far the greatest activation.

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THE INFLUENCE OF ROENTGEN RAYS ON THE ACID-BASE EQUILIBRIUM

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Numerous investigations of the influence of Roentgen rays on the chemical properties of the blood have been made with conflicting results. These differences may be ascribed to some extent to the variations in the quality and quantity of the radiations used and the type of biological system employed. The results found in humans may have been influenced by the different diseases presented by the individuals at the time of irradiation. In view of these conflicting results, it was thought that an investigation of the acid-base equilibrium of healthy adult animals would be instructive.

No attempt will be made to give an exhaustive review of the literature and only those papers which seem pertinent will be mentioned. Hussey (11) found, after irradiating the abdomen of rabbits with unfiltered Roentgen rays of relatively long wavelengths, a rise in the pH and bicarbonate of the plasma. Mahnert and Zacherl (14) showed that the CO₂ content of the blood, taken from patients immediately after irradiation, was reduced. The individuals used in his experiments were being treated for various diseases. Kroetz (13) found an immediate decrease in the pH and CO₂ content of the blood of individuals being treated with the Roentgen rays. Later both the pH and CO₂ rose. Hirsch and Petersen (10) obtained essentially the same results in their investigations. Doub, Bolliger, and Hartman (4) were unable to demonstrate any change in the CO₂ content of dog blood although they did find a decrease in the concentration of hydrogen ions. Golden (6) found no change in the bicarbonate content of either

* Holder of Sterling Research Fellowship at Yale University, 1929-30.

dogs or patients following irradiation. Kolta and Förster (12) observed a constant decrease in the CO_2 of the blood of patients after exposure to the Roentgen rays. Davy (3) was unable to find any change in CO_2 or hydrogen ion concentration. Pohle and Sevringhaus (19), in their recent paper, review the literature concerning the changes in nitrogen and chloride content of the blood after irradiation with the Roentgen rays. They were unable in their investigations to confirm the changes in these constituents which some of the preceding investigators had reported. Doub, Bolliger, and Hartman (4) found that the inorganic phosphorus content of the serum fell after irradiation. The concentration of base in the serum or blood after exposure to the Roentgen rays does not appear to have been determined.

Methods

The animals used in this investigation were normal adult dogs, and monkeys (*Macacus rhesus*). The dogs were fed on a standard diet. This diet was made up of a mixture described by Cowgill (1) as "Dog Diet No. III" which he has shown to be adequate with respect to proteins, salts, and vitamin A. In addition, vitamin B, shown by Cowgill (2) to be necessary in maintaining the nutrition of dogs, was provided in the form of 1.0 gm. of Squibbs' Vitavose daily per kilo of body weight. The bulk of the diet was composed of dog biscuit. Water was liberally supplied. All food was removed from the dog cages the night before blood was taken. The blood was removed from the left ventricle by cardiac puncture, care being taken to be sure that the needle was in the left and not in the right ventricle.

The technique and calculations used in handling the samples of blood obtained were the same as those described by Oard and Peters (16) with the exception that many of the chloride determinations were done by the Hald (7) modification of the micro method described by Patterson (17). No determination of the pH of the serum in these animals was made; hence, a pH of 7.35 was assumed for purposes of calculation.

If the pH of these experiments did not remain constant, as was assumed, and as Davy (3) reported, but varied as other investigators have shown, no significant change in the results would be caused except that if the pH were lowered, the figures for the

bicarbonate, serum protein, and phosphorus would be slightly too high. This would make the figures for the undetermined acids higher. If the pH were higher than the one assumed, the reverse would be true. Hence if the pH varied, as one would be led to believe by the work of previous investigators, there would be a greater difference in the undetermined acids before and after irradiation.

The monkeys were placed on a standard diet. This diet was composed of one banana and a half loaf of bread soaked in 250 cc. of whole milk daily. They were kept on this diet for 2 weeks before bleeding. About 15 cc. of blood were removed from the heart under oil by cardiac puncture. This blood was treated like that of the dogs except that defibrinated blood was used for the non-protein nitrogen determinations.

The dogs were irradiated over the upper abdomen by a Coolidge air-cooled tube. This tube was capable of continuous operation at 180 kilovolts and 10 milliamperes. The dosage factors were as follows: 135 kilovolts, target distance 44 cm., 8 milliamperes, time 37.5 minutes, filters 1.0 mm. of aluminum and 0.5 mm. of copper. In the case of Dog 2 the copper filter was removed.

The monkeys were irradiated with a water-cooled Coolidge tube capable of continuous operation at 200 kilovolts and 50 milliamperes. The target was centered over a point midway between the umbilicus and the xiphoid process. However, because of the small size of the animals, all of the abdomen and the lower part of the chest were exposed to the cone of irradiation. The monkeys while being irradiated were in a box measuring $80 \times 80 \times 180$ cm., lined with 6 mm. of lead. The dosage factors were: 135 kilovolts, target distance 44 cm., 10 milliamperes, time 12 minutes, filters 1.0 mm. of aluminum and 0.5 mm. of copper.

Both the dogs and the monkeys were given 0.4 cc. per kilo of a 10 per cent solution of sodium amytal prior to irradiation.

Results

The concentration of the total base was found to be lowered in both the dogs and the monkeys. This is shown in Charts 1 and 2, the determination just prior to irradiation being taken as the zero point. Dog 1 is charted separately for each irradiation.

The decrease in total base concentration varied between 4.6

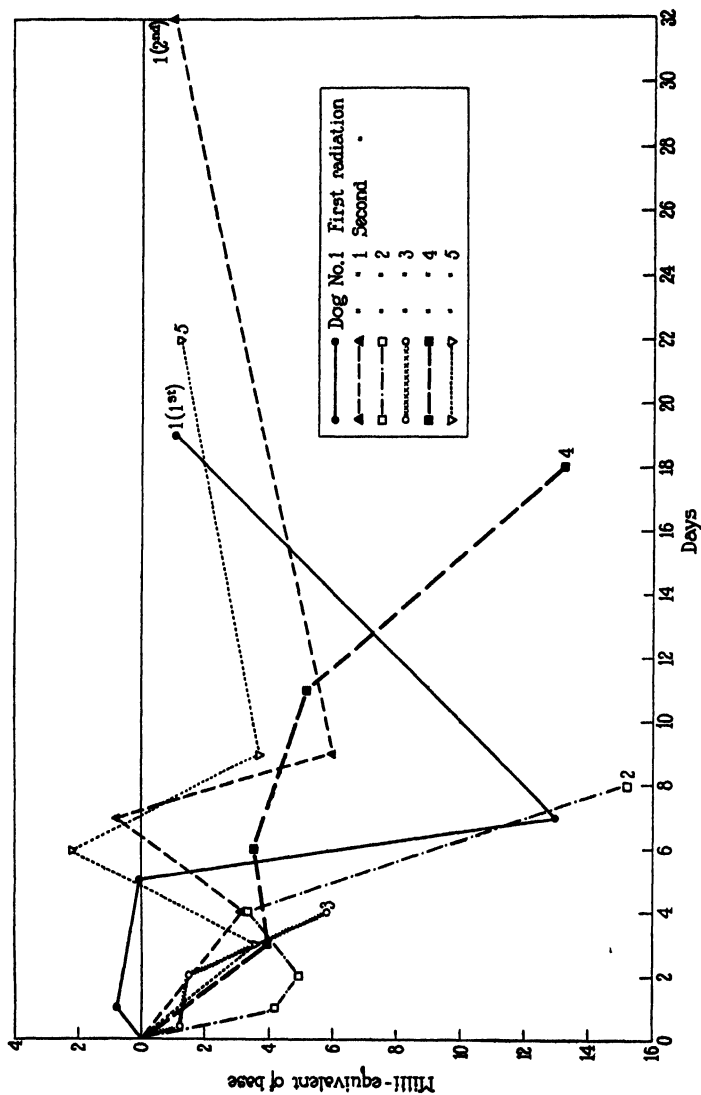


CHART 1. Curves showing loss of base after exposure to Roentgen rays

milli-equivalents in Dog 5 to 12.9 milli-equivalents in Dog 1 with an average decrease of 9.8 milli-equivalents. In giving these averages Dog 2 is not included because of the diarrhea which complicated the picture. The findings in this dog are discussed below. The decrease in the monkeys was 11.6 and 10.8 milli-equivalents.

There were no significant changes found in the CO_2 , non-protein nitrogen, serum protein, sodium chloride, phosphorus, or cell volume and as these findings are in agreement with those of

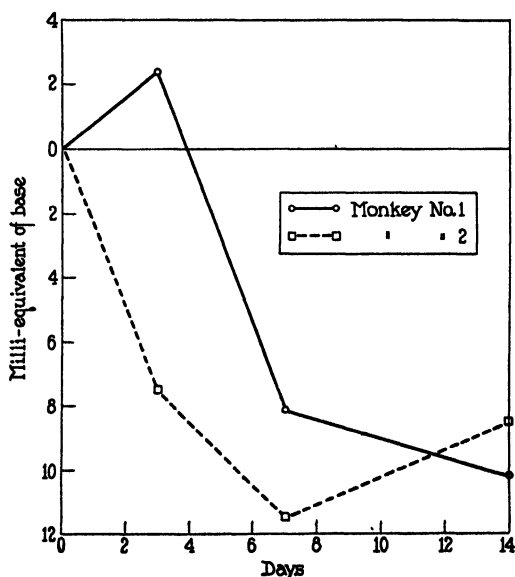


CHART 2

Pohle and Sevringhaus they are not reported in detail here. As there was no significant change in these constituents the acid electrolytes lost were from the undetermined acids.

Brief protocols of the animals are recorded below.

Dog 1—Male collie; weight, 15 kilos. March 12, 1930, to March 17, 1930, well, good appetite. March 13, 1930, irradiated. March 18, 1930, refuses food, listless, no diarrhea. March 19, 1930, to April 6, 1930, well, good appetite. April 7, 1930, Roentgen ray burn of skin on upper abdomen, measuring 1×2 cm. This burn healed in about a week; from this time on

to the time of death the animal remained in good condition. Killed on May 13, 1930. Necropsy failed to reveal any lesions either grossly or microscopically.

Dog 2—Police dog, male; weight, 17 kilos. March 24, 1930, to March 27, 1930, well, good appetite. March 26, 1930, irradiated. March 28, 1930, listless, appetite poor. March 29, 1930, to time of death on April 21, 1930, appetite poor, profuse foul smelling diarrhea. Marked loss of weight. Weight at time of death 12 kilos. Necropsy was unsatisfactory as the animal died in the middle of the night and the postmortem examination was not made until the next morning.

Dog 3—Female airdale; weight, 14 kilos. April 8, 1930, to April 10, 1930, well, good appetite. April 8, 1930, irradiated. April 11, 1930, slight upper respiratory infection. April 12, 1930, eyes watery, appetite fair. April 13, 1930, slight diarrhea, eyes watery, appetite poor. April 13, 1930, to April 15, 1930, condition remained the same. April 15, 1930, succumbed to cardiac puncture. Necropsy revealed a pharyngitis from which streptococcus hemolyticus was isolated. No other lesions were found.

Dog 4—Pregnant female police dog; weight, 16 kilos. April 29, 1930, irradiated. Dog remained well throughout the experiment. A necropsy performed on May 20, 1930, was negative.

Dog 5—Male bird dog; weight, 12 kilos. May 27, 1930, irradiated. This dog remained well throughout the experiment and was killed on July 2, 1930. Necropsy revealed no lesions.

Monkey 1—Male, weight 2400 gm.

Monkey 2—Female, weight 2200 gm.

Both monkeys remained well throughout the experiment. Their appetites were good and they had no diarrhea. No necropsies were performed.

DISCUSSION

From the results stated above it would appear that the decrease in the concentration of total base is not due to a hydremia of the blood as there is no significant change in either the serum proteins or the cell volume.

As the decrease in total base in most instances exceeds the total concentrations of Ca, K, or Mg ions, it is logical to assume that the sodium ions are the ones affected. Sodium does not exist in the body in a solid form; hence if its concentration in the serum is decreased, it is probably excreted.

Another feature of the reduction of base which is different from the reductions reported in other conditions is that the acid electrolytes which are lost are not bicarbonate but are among the undetermined acids. These are also responsible for part of the de-

crease in the acid electrolytes in pregnancy (16). It is impossible at present to say what the nature of this loss is.

In the case of Dog 2 the failure to filter out the radiations of relatively long wave-length produced marked toxic symptoms with diarrhea and a larger diminution of base together with a rise in the non-protein nitrogen and serum protein, the result being similar to that found by Hall and Whipple (8).

Although the ionic concentration of so complex a material as the blood is not an exact measure of osmotic pressure, the two are probably closely related. This was pointed out by Oard and Peters (16) and by Sunderman, Austin, and Camack (20). The importance of maintaining this concentration is apparent from the serious results which immediately supervene when the concentration is changed, as occurs in vomiting (5), diarrhea (9), and miners' cramps (15). There are, however, three other conditions—pregnancy (16), pneumonia (20), and nephrosis (18)—in which there is a decrease in the total ionic concentration. The first, pregnancy, is in many instances unaccompanied by any impairment of the woman's well being.

In the experiments here described there is added a fourth condition in which ionic concentration of the organism is changed. These conditions, pregnancy, pneumonia, nephrosis, and exposure to the Roentgen ray, differ from the others, vomiting, diarrhea, and miners' cramps, in that dehydration is not a pronounced feature. Therefore, it would seem that a change in the ionic concentration is possible when not accompanied by any depletion of the body water.

In conclusion, mention will be made of certain experiments of which circumstances have not permitted the completion. The experiments of Hussey (11) on rabbits were repeated on three rabbits with the exception that total base was determined instead of pH. No change was found in the bicarbonate and total base. The time of exposure was then increased from 15 to 20 minutes. Three rabbits treated in this fashion showed a rise in the bicarbonate similar to that found by Hussey, and also an increase in total base. Also one dog was irradiated for 45 minutes with the following dosage factors: 100 kilovolts, target distance 44 cm., 5 milli-amperes, filters 1.0 mm. of aluminum and 0.5 mm. of copper.

Following irradiation the bicarbonate and chloride were found unchanged but the total base remained elevated for 2 weeks. While these experiments are too brief to be conclusive, they tend to show that there is a definite species difference in the response of rabbits and that of dogs and monkeys to the Roentgen ray. Furthermore, the organism may fail to respond, or respond in different fashion, to various qualities in the irradiations.

SUMMARY

An investigation of the bicarbonate, chloride, protein, phosphorus, total base, and non-protein nitrogen concentration of the serum, and cell volume was made on five dogs and two monkeys (*Macacus rhesus*) before and after irradiation with the Roentgen rays.

A consistent decrease in the concentration of the total base and undetermined acids was found in the dogs and monkeys following irradiation. This decrease was in some cases preceded by an increase. There was no change in the other constituents studied.

The decrease in the concentration of base is probably due to an excretion of sodium.

Mention is made of the fact that under certain conditions rabbits may respond to Roentgen ray irradiation by an increase in bicarbonate and total base and that dogs also may respond by an increase in total base concentration rather than by a decrease.

The author wishes to express his appreciation to Dr. J. P. Peters for his interest and assistance in this work.

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BENEFICIAL EFFECTS OF FAT IN HIGH SUCROSE DIETS WHEN THE REQUIREMENTS FOR ANTINEU- RITIC VITAMIN B AND THE FAT-SOLUBLE VITAMIN ARE FULLY SATISFIED*

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INTRODUCTION

In a previous communication (1), we reported experiments showing definitely that the presence of dietary fat reduces the body requirement for the water-soluble antineuritic vitamin B. In the total absence of added vitamin B, it was necessary to add as much as 50 per cent fat markedly to improve such animals as contrasted with their "fat-free" controls. The higher we raised the dietary vitamin B level, the less was the need for dietary fat, yet it was clear that at the level of 800 mg. of yeast daily, the rat organism still responded to the addition of fat. Were we to conclude that a high level of vitamin B, such as 800 mg. of brewers' yeast, does not entirely satisfy the vitamin B requirements of the rat on a "fat-free" diet?

It was proper for us to reject the notion that we were dealing with some peculiar effects of a fat *versus* a fat-free diet until such a time as we could show that, even with exceedingly abnormal high levels of vitamin B, fat was still able to improve the diet.

We should emphasize that our "fat-free" diets were not entirely fat-free, neither the casein nor the yeast having been treated with fat solvents. Moreover, 2 drops of cod liver oil were found in this

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laboratory to prevent development of the peculiar deficiency due to the complete exclusion of fat, noticed simultaneously and independently by McAmis, Anderson, and Mendel (2), and Burr and Burr (3), and described in greater detail by Burr and Burr (4).

EXPERIMENTAL

Female rats, started on the experimental diets when 21 days old, were reared in individual cages on raised wire screens to prevent access to their feces.

TABLE I
Composition of Diets Used

All diets were supplemented with 2 drops of cod liver oil daily.

Diet No.	"Fat-free" diet	Diets containing fat			
	542	569	590	593	594
Casein LIII*	20.0	30.0	30.0	30.0	30.0
Autoclaved yeast*	10.0	10.0	10.0	10.0	10.0
Sugar	70.0	41.0	41.0	41.0	41.0
Salt Mixture 185	4.0	4.0	4.0	4.0	4.0
"Synthetic" cottonseed oil		25.0			
Lard			25.0		
Coconut oil				25.0	
"Synthetic" coconut oil					25.0

* Casein LIII is washed 1 week with acidulated water and then dehydrated with alcohol and ether. Autoclaved yeast is Fleischmann's yeast autoclaved for 6 hours at 18 to 20 pounds pressure. We wish to express our gratitude to the Fleischmann Laboratories of Standard Brands Incorporated for this excellent source of the heat-stable vitamin.

The basal "fat-free" diet used in this work was Diet 542 plus 2 drops of cod liver oil daily. Fat diets all carried 25 per cent of fat, with the sugar and casein levels adjusted to maintain approximately the same nutritive ratio (Table I).

Lard and coconut oil were the natural fats used. "Synthetic" coconut and cottonseed oils were prepared as previously described (1) by saponification with 20 per cent alcoholic potash, collecting and washing the fatty acids, and distilling and esterifying them with redistilled glycerol. As sources of vitamin B we used brewers'

yeast,¹ ether-extracted wheat germ and rice germ, and alcoholic extracts of rice bran and rice polish.² The rice bran, representing the outer layers of the rice kernel, was extracted with 25 per cent alcohol (1 kilo of rice bran to 4 liters of alcohol) and concentrated *in vacuo* so that 1 cc. = 4 gm. of rice bran. This we labeled Extract 8-M. The rice polish extracts represent the product obtained from the rice polish by a procedure identical with that used to obtain the rice bran extracts. The rice polish extract we termed Extract 8-C. 0.5 cc. of either extract was found to satisfy the rat requirement for vitamin B.

Three series of experiments were carried out. In each of these series, the vitamin B was fed at levels satisfying many times the maximum vitamin B requirements of the rat. Series I is outlined in Table II.

TABLE II

Basic diets	Sources of vitamin B
Diet 542, "fat-free"	1.5 gm. ether-extracted wheat germ
" 590, 10 per cent lard	1.5 " " rice "
	3 cc. Extract 8-M
	3 " " 8-M plus 1.4 gm. ether-extracted wheat germ

In this series four animals were used in each group. The results are presented in Fig. 1. Only composite curves are shown since they reflect rather accurately the behavior of the groups. In the absence of added fat, the growth of the rat is about the same regardless of the source or the amount of vitamin B administered. The addition of lard to the diet brings about a uniformly greater growth in all the groups. The surprising feature of this series is found when the growth obtained on Diet I³ is compared with that

¹ A whole dried brewers' yeast, generously furnished by the Vitamin Food Company through Dr. Edward A. Rumley.

² The rice bran and rice polish were kindly donated by Rosenberg Brothers of San Francisco.

³ Diet I (our normal stock diet) consisted of whole wheat, 67.5; casein, 15.0; whole milk powder, 10.0; sodium chloride, 1.0; calcium carbonate, 1.5; and milk fat, 5.0.

of the experimental groups. The rats on Diet I are not superior to those on "fat-free" diets and are definitely inferior to the rats receiving lard. Superiority of animals on diets composed of isolated relatively pure foodstuffs when compared with those on diets of natural substances (*e.g.* Diet I) is very rarely encountered.

The results of the first series indicated that fat in the diet had a favorable influence on growth which could not very well be explained by any relation to vitamin B. We desired to know if this

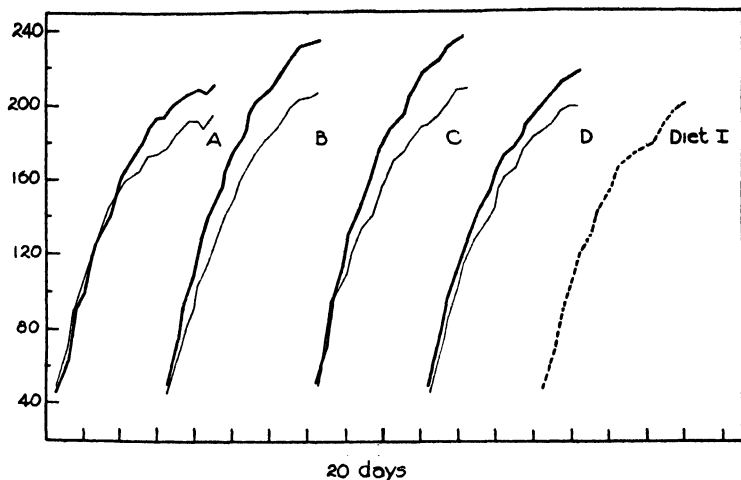


FIG. 1. Composite growth curves. Four animals in each group. The heavy line indicates the growth of the animals receiving a diet containing 25 per cent lard (Diet 590). The light line depicts the growth of the animals receiving a "fat-free" diet (Diet 542). Both diets were supplemented with large amounts of vitamin B in the following order: Curve A, 3 cc. of Extract 8-M; Curve B, 1.5 gm. of ether-extracted wheat germ; Curve C, 1.5 gm. of ether-extracted wheat germ and 3 cc. of Extract 8-M; Curve D, 1.5 gm. of ether-extracted rice germ. Diet I is the normal stock diet.

superiority was conferred by fat *per se*, or by some impurity in the fat. We therefore planned another series of experiments in which 25 per cent of "synthetic" cottonseed oil was used (Diet 569). As sources of vitamin B, we used 1.5 gm. of ether-extracted wheat germ, 1.5 gm. of brewers' yeast, and 3 cc. of Extract 8-M. Each group consisted of four animals. The results are shown in Fig. 2.

Unfortunately we were not aware of the rapidity with which

"synthetic" cottonseed oil becomes rancid. Early in the experiment the rats declined rapidly in weight and well being, and we were greatly puzzled until we traced the difficulty to rancidity in the

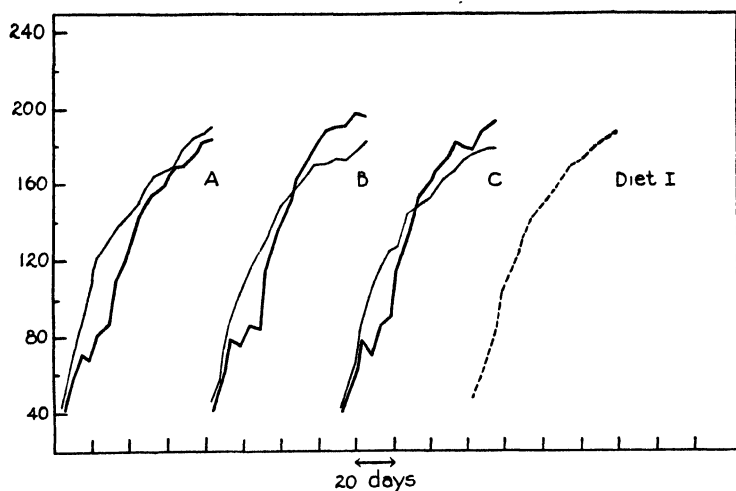


FIG. 2. Composite growth curves. Four animals in each group. The heavy line indicates the growth of animals receiving a diet containing 25 per cent "synthetic" cottonseed oil (Diet 569). The light line depicts the growth of animals receiving a "fat-free" diet (Diet 542). Both diets were supplemented with vitamin B in the following order: Curve A, 1.5 gm. of ether-extracted wheat germ; Curve B, 1.5 gm. of brewers' yeast; Curve C, 3 cc. of Extract 8-C. Diet I is the normal stock diet.

TABLE III

Basic diets	Sources of vitamin B
Diet 542, "fat-free"	3 cc. Extract 8-M
" 590, 10 per cent lard	3 " " 8-C
" 593, 10 " " coconut oil	
" 594, 10 " " "synthetic"	
coconut oil	

"synthetic" cottonseed oil. One rat died due to the toxicity of the rancid material (5) before we instituted corrective measures. These consisted in making up the oil diets fresh every other day. The animals promptly resumed their growth and apparently recov-

ered entirely, in spite of initial stunting due to rancidity. From an inspection of Fig. 2, we see that in the presence of much brewers' yeast or abundance of Extract 8-C, fat still confers definite benefits on rats, as may be seen by comparing them with sisters on these same materials but with a "fat-free" diet.

A third and final series of experiments employing larger numbers of animals was now designed to settle the question whether fat still

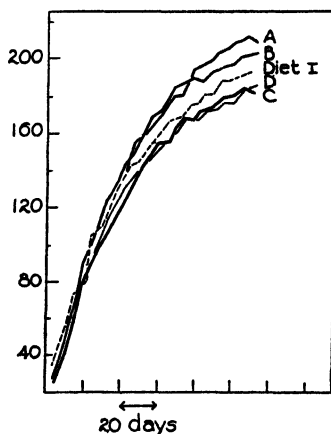


FIG. 3. Composite growth curves. Twelve animals were used in the groups represented by Curves B, C, and D; eleven in the remaining two groups. The source of vitamin B was 3 cc. of Extract 8-C. The heavy line depicts the growth of animals on a diet containing 25 per cent fat. Curve A, coconut oil (Diet 593); Curve B, lard (Diet 590); Curve C, "synthetic" coconut oil (Diet 594). The light line, Curve D, depicts the growth of animals on the fat-free diet (Diet 542). Diet I is the normal stock diet.

exerts a beneficent influence after the vitamin B requirements have been fully satisfied; it is outlined in Table III.

Twelve animals were put in each group. Fig. 3 shows the growth curves of the rats receiving the various basic diets supplemented with 3 cc. of Extract 8-C as a high level of vitamin B. It is readily seen that the natural coconut oil and lard are definitely superior to the "fat-free" diet.

Fig. 4 depicts the growth of those animals receiving 3 cc. of Extract 8-M as the source of their vitamin B. This series was allowed to run for about a year. At 245 days of age, one-half of the rats of

each group received the non-saponifiable fraction of wheat germ oil equivalent to 20 drops of the oil. A difference in growth (6) did not result from this supplement. The animals on the "fat-free" diet showed no marked deficiencies at the end of this time, but were definitely inferior to those rats receiving fat in their diet

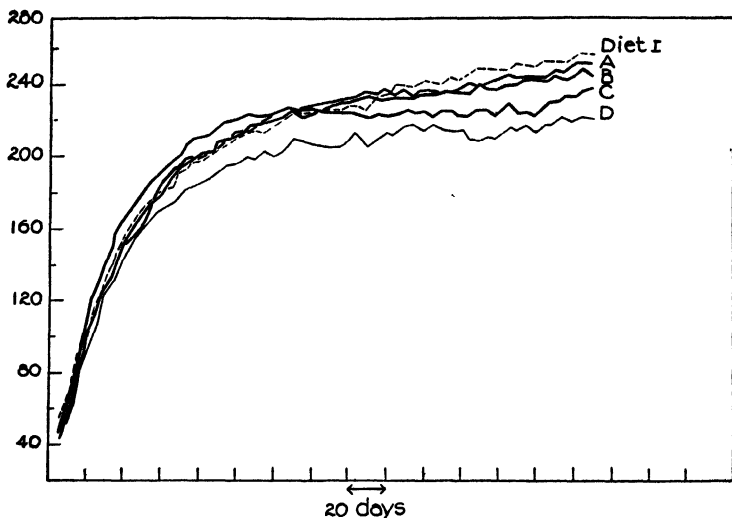


FIG. 4. Composite growth curves. Ten animals were used in the groups represented by Curves A and D, eleven animals in that of Curve B and for Diet I, and twelve animals in the group represented by Curve C. The source of vitamin B was 3 cc. of Extract 8-M. The heavy line depicts the growth of animals maintained on a diet containing 25 per cent fat. Curve A, coconut oil (Diet 593); Curve B, "synthetic" coconut oil (Diet 594); Curve C, lard (Diet 590). The light line, Curve D, demonstrates the growth obtained by animals on the "fat-free" diet (Diet 542). Diet I is the normal stock diet.

(coarser fur, tendency toward skin dryness, and lower body weight). The animals receiving the coconut oils were slightly superior to those receiving the lard. Diet I, though somewhat inferior to the fatty diets in the early part of the experiment, showed steady improvement relative to the other diets, and at the end of the experiment was slightly, but definitely, superior.

CONCLUSIONS

"Fat-free" diets which are no longer improved by the addition of vitamin B can nevertheless be slightly improved by the addition of fat.

That this improvement is not due to fat-soluble vitamins or other impurities carried in the fat, is indicated by the fact that our synthetic fats were able to improve fat-free diets as well as did natural fats; furthermore the vitamins A, D, E, and the substance F were abundant in all diets.

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STUDIES ON THE RELATION OF MANGANESE TO THE NUTRITION OF THE MOUSE*

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There are a number of minor inorganic elements constantly found in animal tissues whose function, or even necessity, is still in question. Among such elements are manganese, zinc, cobalt, and nickel. In the last few years the necessity for copper as a supplement to iron in hemoglobin building has been firmly established (1). After this relationship had been demonstrated a number of investigators reported results indicating that manganese could also function like copper in hemoglobin building. Our own work (2), as well as the work of Krauss (3), Lewis *et al.* (4), and others, has not confirmed these claims. In fact, Titus, Cave, and Hughes (5), who first announced this possible relation of manganese to hemoglobin synthesis, have been unable to confirm their earlier results (3).

While it may be admitted, therefore, that manganese has no relation in the mammal to hemoglobin building, yet its constancy in animal tissues (6) makes it very probable that it does perform some essential function. Recent work by Richards (7) indicates a wide distribution of manganese in both plant and animal materials with a special accumulation of this element in the reproductive organs. While Richards makes no claims to the indispensability of manganese for normal growth and development, yet she considers it very possible that manganese may have a significant physiological action, particularly in relation to the normal performance of the reproductive organs.

In order to supply direct experimental evidence that manganese

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is an essential element for normal metabolism, the animal must be fed a diet complete in all other factors and deficient only in manganese. Most of the workers studying the importance of this element have used basal rations which have contained considerable amounts of manganese or which have been lacking in other important factors. McCarrison (8), using a diet which was not completely devoid of manganese, found the addition of this element in proper concentrations to exert a markedly favorable influence on the growth of rats. Richards (7) fed farm animals on diets low in manganese and detected no change in growth or general health, but the foodstuffs used probably contained large enough amounts of the element to satisfy the animal's requirements. Bertrand and coworker (9) used diets highly purified in respect to manganese but deficient in the vitamins, and therefore the mice placed on these diets rarely survived for periods longer than 35 days. It is our purpose in this paper to present results which have been obtained by the use of diets which furnish all the recognized nutritional factors, but which are exceedingly low in manganese.

The white mouse was chosen as the experimental animal because if highly purified "synthetic rations" are to be used it is desirable to conserve as much as possible the amount of food material needed for each animal. We have found it possible to make preparations of casein, certain sugars, salts, and sources of vitamins A and D which are practically manganese-free but have as yet been unable to prepare a vitamin B complex which is low in this element. Although work on this preparation is being continued, we have turned, in lieu of the synthetic ration, to milk as the possible food medium. Milk is extremely low in manganese, containing approximately 0.02 mg. per liter, and still furnishes most of the necessary food factors with the exception of iron and copper. It is difficult to assemble a synthetic diet which is lower in manganese than a corresponding amount of milk, even when only the most highly purified constituents are used. We feel that further work on the demonstration of the importance of manganese does not depend so much upon the preparation of diets which contain less manganese than milk but upon greater care of the experimental animal, *e.g.* regulation of the amount of manganese in the animal at birth and prevention of manganese contamination both during the the suckling period and after the animal is placed on the experimental diet.

EXPERIMENTAL

The stock colony of mice was kept on shavings and fed the rat colony stock ration and whole milk *ad libitum*. The females were removed from the colony shortly before parturition and placed on screens in individual cages. A part of the screen was covered with a thin board upon which was placed a roll of cotton in which the young could be born. The mother was continued on the stock ration and milk until the young opened their eyes, usually 10 to 12 days, when the dry ration was removed allowing the mouse access to milk alone. In some cases the mother was given no dry food in the cage with the young but removed to a separate cage for feeding and returned to the young for nursing after being thoroughly brushed to remove any adhering feed. This procedure was for the purpose of minimizing the possibility of the mother carrying to the young any of the stock ration, and thus to reduce the danger of contact with manganese.

When the young were 17 to 18 days old the mice, together with the mother, were transferred to glass cages provided with suitable glass grids to prevent all possible manganese contamination. In these cages the animals were supplied with whole cow's milk alone. The young mice were generally weaned at 21 days of age, but in a few cases where the mice did not consume the cow's milk readily the mother was left with them for 2 or 3 days longer. When the young were well accustomed to the cow's milk each litter was divided into two equal groups. One group received the milk supplemented with iron and copper in quantities sufficient to supply each mouse 0.15 mg. of Fe and 0.01 mg. of Cu daily. The other group received the same diet except that sufficient MnCl_2 was added to supply each mouse 0.01 mg. of Mn daily. In some cases the two groups from the same litter were supplied with a mixture, consisting of casein 20 per cent, lactose 75 per cent, and butter oil 5 per cent, which was practically manganese-free. This was fed *ad libitum* as a dry mixture, and merely supplemented the milk ration.

Previous work with rats (10) has shown that whole milk supplemented with iron and copper alone serves very well for fairly good growth and normal maintenance of the animal, but that the reproductive performance is below normal. No distinct pathological conditions, except those associated with the reproductive organs,

seem to arise on a whole milk, copper, and iron diet. Consequently we were limited in our observations on the effect of manganese to such phenomena as rates of growth and ovulatory rhythm. In a few cases a study of the oxygen uptake of the tissues through the use of the Barcroft apparatus was made, but such studies are as yet incomplete and preliminary and will not be dis-



CHART 1. Growth curves of three male mice from Litter 5. The young together with the mother were placed in glass cages when 17 days old, weaned at 21 days of age, and started on the milk, iron, and copper ration when 24 days old. Mice 21 and 22 received no manganese. Mouse 20 received 0.01 mg. of manganese daily.

cussed in this paper. No attempt was made in the early work to keep accurate consumption records for the different mice. We can say that there were no large differences in the amount of milk consumed by the manganese and non-manganese groups. No doubt accurate consumption records would aid in deciding whether the manganese effect was due to a greater food consumption or to a better utilization of the food that was consumed.

The growth records of a few typical animals are given in Charts 1 and 2. The records in each chart are for mice which were litter mates and of the same sex, the only difference being the addition

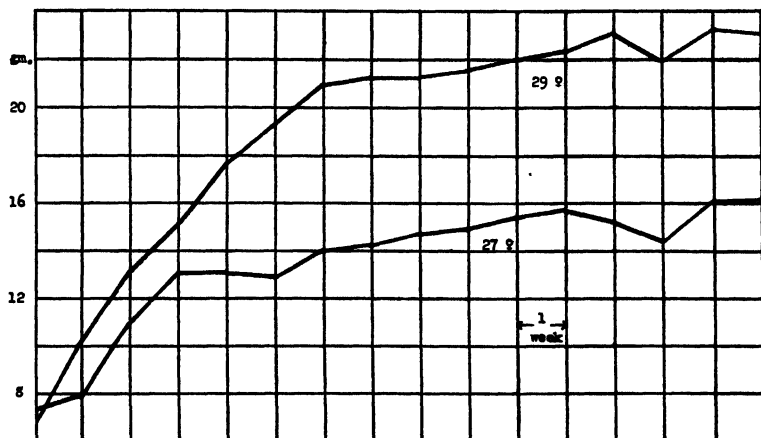


CHART 2. Growth curves of two female mice from Litter 11. The young together with the mother were placed in glass cages when 18 days old, weaned at 21 days of age, and started on the milk, iron, and copper ration when 26 days old. Mouse 27 received no manganese. Mouse 29 received 0.01 mg. of manganese daily.



FIG. 1. The effect of manganese on growth. Mouse 39 (right) received whole cow's milk reinforced daily with 0.15 mg. of Fe as FeCl_3 , 0.01 mg. of Cu as CuSO_4 , and 0.01 mg. of Mn as MnCl_2 . Mouse 37 (left) received the milk reinforced with similar amounts of copper and iron only. Both are females from the same litter and had been on the experimental ration 7 weeks when photographed. Weight, Mouse 39, 19.1 gm.; Mouse 37, 12.7 gm. These are typical results.

of manganese. The weight records were started when the mice were placed on the experimental diet, and the mice were weighed weekly thereafter. It is apparent from these charts, and many

other records which due to lack of space cannot be published, that manganese has a measurable effect upon growth (see Fig. 1) and that the effect is sufficiently consistent to warrant the belief that manganese is indispensable for normal growth.

Just what particular tissue or tissues are involved in this accelerated growth is not disclosed by these experiments, but the results obtained from the study of the ovulatory rhythm indicate that manganese *is closely connected with the reproductive organs*. The ovulatory rhythm of all the female mice on the experimental diets was studied by taking vaginal smears daily according to the method used by Long and Evans (11) for rats. The vaginal

TABLE I
Effect of Manganese on the Frequency of Estrus in Mice

No manganese					With manganese				
Mouse No	Period of observation			No of estrous cycles	Mouse No	Period of observation			No of estrous cycles
	Age when started	Age when concluded	Length of time			Age when started	Age when concluded	Length of time	
	days	days	days			days	days	days	
24	93	156	63	0	23	93	156	63	8
12	68	142	74	2	16*	68	142	74	7
27	57	116	59	0	29	57	116	59	6
33	55	94	39	0	36	55	94	39	4
37	61	92	31	0	39	61	92	31	3

* Mouse 16 became pregnant and gave birth to five young when 134 days old.

examination was started in most cases when the mice were 7 to 9 weeks old. The records of the estrous cycles of a few of the females which were on the manganese-low diet and of their litter mates which were given the manganese supplement are given in Table I. The period during which these observations were made is also given for each animal.

It is readily seen from Table I that the number of estrous cycles is greatly reduced in the mice limited to a manganese-low diet. In four of the five females recorded no signs of ovulation could be detected during the period of observation, while in the fifth two estrous cycles were noted. In all of the animals studied, which

were restricted to the low manganese diet, we have never observed more than two estrous cycles during periods of 10 weeks or longer. When the diet is supplemented with a trace of manganese, the number of estrous cycles is decidedly increased in all animals. The frequency of the cycles appears to be approximately normal. Allen (12) states that the average duration of the cycles in mice is 4 to 6 days. If the average duration of each cycle is calculated from the figures in Table I the result is greater than 6 days, but many of the mice had not reached sexual maturity when the vaginal smears were started. The interval was never found to be greater than 14 days, which Allen gives as the maximum time between estrous cycles in mice.

These results are substantiated by a few records on reproduction. These records are as yet very meager since we have made no attempts to mate the female mice on the experimental diets with normal males or *vice versa*. However, we have noted that in the groups which contained both male and female mice no pregnancies occurred in the groups on the restricted diet. On the other hand some of the females in the group receiving the manganese mated and gave birth to normal litters of young. These results indicate that manganese is definitely concerned with normal reproduction in the mouse. Waddell, Steenbock, and Hart (10) have reported preliminary experiments with rats which indicate that manganese greatly improves the ovulation rhythm in females on the milk, copper, and iron diet. It is useless at this time to speculate regarding the mechanism by which manganese is active except to suggest that manganese undoubtedly has a distinct influence on the normal activity of the ovary.

SUMMARY

1. The addition of traces of manganese (0.01 mg. of Mn as $MnCl_2$ per mouse daily) to a diet of whole cow's milk supplemented with iron and copper has a favorable effect upon the growth of mice.

2. Mice reared on a diet of whole cow's milk supplemented with iron and copper fail to ovulate normally. Mice reared on a diet of whole cow's milk supplemented with iron, copper, and *manganese* exhibit normal estrous cycles.

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THE INFLUENCE OF THE RATION OF THE COW UPON THE VITAMIN B AND VITAMIN G CONTENT OF MILK*†

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In a previous publication (1) it was shown that milk contains a relatively large amount of vitamin G (B_2) while the amount of vitamin B (B_1) is small. Sherman and Axtmayer (2) in a study of the composite nature of vitamin B found that dried skim milk was apparently richer in vitamin G than in vitamin B. Aykroyd and Roscoe (3), in a similar study, made the observation that milk was a poorer source of the antineuritic vitamin than of the antipellagric factor. In view of these observations it was thought pertinent to study the influence of the ration of the cow upon the relative potency of milk in these two known factors of the vitamin B complex. We were cognizant of the fact that Bechdel and coworkers (4) observed that cows are apparently able to synthesize vitamin B through bacterial action in the rumen. Since Bechdel published his first paper vitamin B has been found to be composed of two or more factors. Whether any other factor besides the antineuritic vitamin is synthesized in the rumen requires further investigation. It is also conceivable that the feed of the animal may influence the type of microorganism present in the rumen and as a result affect the amount of each of these factors of the vitamin B complex which may be synthesized. The present report deals only with the influence of the ration of the cow upon the vitamin B (B_1) and vitamin G (B_2) content of milk.

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† In speaking of the vitamin G content of milk in this paper the combined effect of vitamin G and a third factor, if present in milk, is implied.

EXPERIMENTAL

The technique of determining the two different factors of the vitamin B complex, as well as the general laboratory technique, has been described in a recent paper (5). In this paper the vitamin B preparation or A fraction refers to that part of the acidulated yeast extract which is adsorbed by fullers' earth, or that part of the acidulated extract of rice polishings which is not precipitated by lead acetate, and which apparently contains only the antineuritic vitamin. The B fraction is that part of the extract of yeast which is not adsorbed by fullers' earth. In all of the experiments the rats were kept individually in wire cages with raised floors. The basal diet consisted of casein (purified) 18, starch 64, McCollum's Salt Mixture 185 (6) 4, Crisco 10, agar-agar 2, and cod liver oil 2 parts.

The milk used was obtained from cows in the Experiment Station herd. The regular ration consisted of a grain mixture of corn 400, oats 300, bran (wheat) 100, and linseed oil meal 100 parts, and, as roughage, alfalfa hay and corn silage. When the cows were turned out to pasture the grasses were substituted for the alfalfa hay and silage. During the first part of the pasture grass feeding period the amount of the grain mixture fed daily was reduced below that fed during the dry feeding period, but this was gradually increased until the amount of grain fed was about equal in both periods.

The data in this paper were obtained as the result of two experiments: first, a comparison of pasture grass *versus* dry feed as to their effect on the vitamin B and vitamin G content of milk; second, the influence of the stages of maturity of pasture grasses upon the vitamin B and vitamin G content of milk. The vitamin B and vitamin G content of the pasture grasses was also investigated. The flora of the pasture grasses for both experiments consisted principally of blue-grass, orchard grass, and white clover. The grasses were dried in the attic of the laboratory building, ground to a fine powder in a ball mill, and fed on an air-dried basis. In the first experiment the milk was collected daily and fed fresh, while in the second experiment the milk was pooled, separated, dried before an electric fan at a low temperature (32-45°) and fed as dried skim milk. The first experiment was conducted during the summer of 1929, the second during the spring and summer of 1930.

Pasture Grass versus Dry Feed—The curves in Chart 1 are composite growth curves of four rats each, and show the vitamin B and vitamin G content of the milk from cows on pasture and on dry feed. No attempt was made to differentiate between the growth factor and the antipellagric factor. The composite growth curves of the rats on the separate vitamin fractions, as well as those of the basal control group, are also given. This chart indicates that the vitamin G content of the milk is influenced by the ration of the cow and in this case substituting pasture grass

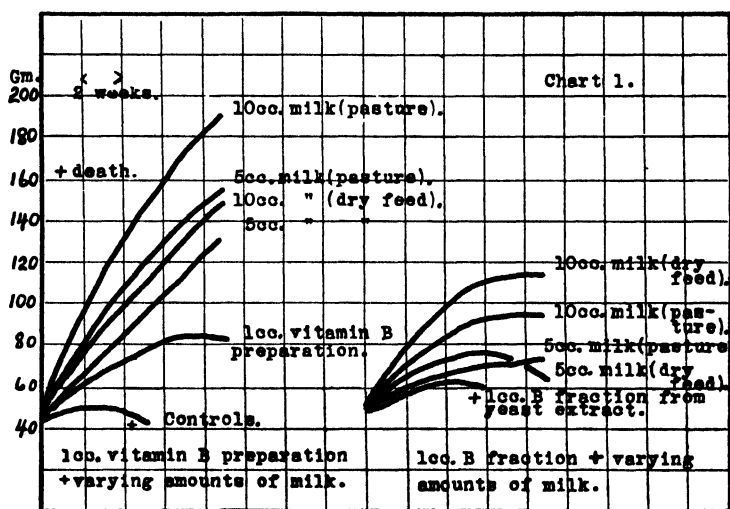


CHART 1. Composite growth curves, each for four rats, comparing the vitamin B and vitamin G content of milk from cows on pasture and on dry feed.

for hay produced milk with twice the amount of vitamin G as that produced on dry feed. It also shows, as is now well known, that milk is relatively low in vitamin B. 5 cc. of milk have been found to be enough to supply sufficient vitamin G for normal growth, whereas Osborne and Mendel (7) and Krauss (8) found that about 16 cc. are necessary to supply the vitamin B requirements for normal growth. This gives a ratio of antipellagric to anti-neuritic vitamin of 3:1. These curves indicate that dry feed, consisting of the grain mixture mentioned elsewhere, alfalfa hay,

and corn silage, produces milk higher in vitamin B than does a ration including pasture grasses and grain (lower amount of grain mixture per cow than when on the dry feed).

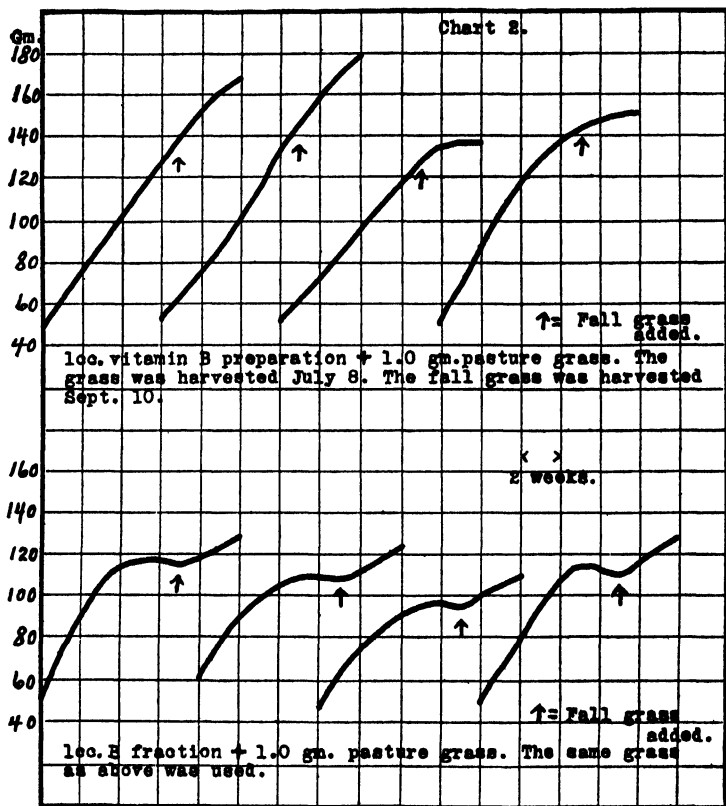


CHART 2. Composite growth curves obtained from feeding pasture grasses.

The growth curves shown in Chart 2 record the results obtained from feeding the dry finely ground pasture grasses, and show that pasture grass is relatively high in vitamin G and contains only a fair amount of vitamin B. The results also show that mature grass is slightly lower in vitamin B than is fresh tender grass. The grass used in the first part of the experiment was collected on

July 8, while the grass used in the last part of the experiment was collected on September 10. In looking for a possible

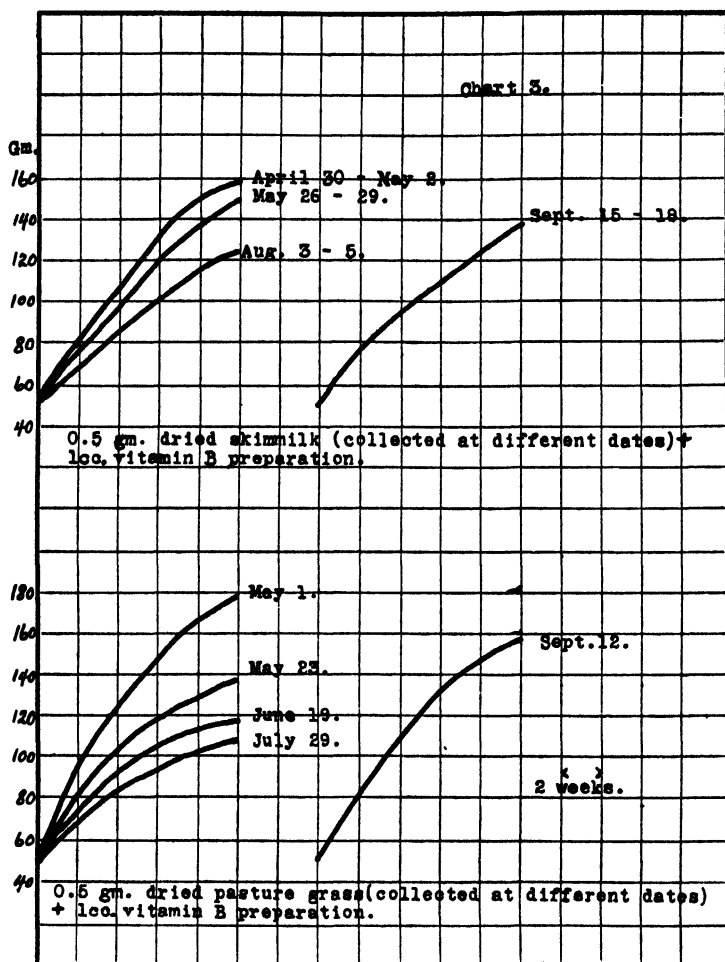


CHART 3. Composite growth curves of rats receiving milk and grass collected at different dates throughout the summer.

explanation as to why, when the grass collected on September 10 was added to the diet, there was a stimulation of growth where vitamin B was the limiting factor, one naturally turned to the

weather reports. It was found that June and early July were rather cold and damp, which is not conducive to the rapid growth of grass, whereas August was dry, allowing for an accumulation of nitrates in the soil. Several rains, with high temperature, were experienced shortly before the grass was collected in September. These weather conditions, as they affect the growth and nutritive value of grass, may account for the growth curves as recorded in Chart 2.

Influence of Maturity of Grasses—The second experiment in this series was conducted in order to repeat the first trial and determine the effect of pasture grasses of different maturities on the vitamin B and vitamin G content of milk. In this case, the milk was collected from four cows for 3 days before they were turned out to pasture (May 3). Further samples of the milk and

TABLE I
Analyses of Pasture Grasses

Date	Moisture	Protein	Ash
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
May 1.....	9.81	20.31	8.40
“ 23.....	8.13	14.37	8.06
June 19.....	7.51	10.06	6.85
July 29.....	7.78	9.81	8.40
Sept. 12.....	11.06	16.06	9.43

grass were collected at different 3 day periods throughout the summer, as indicated in Chart 3. The samples were prepared as previously described and fed separately and daily to groups of four rats each. Chart 3 shows the composite growth curves of rats receiving the milk and grass collected at different dates throughout the summer. The summer of 1930 was noted for being the driest year on record and possibly the differences in the grasses are accentuated somewhat from that of a normal year. As a rule hays and grasses are low in vitamin B and rather high in their vitamin G content. The data obtained in this study (Chart 3) give evidence that the vitamin G content of grasses decreased as the season or drought advanced. With the approach of fall rains the vitamin G content again increased to amounts comparable to those found in spring or early summer grasses. Milk obtained

during the pre-pasture period was as high if not higher in vitamin G content than that of the early pasture period. This may be accounted for by the fact that the cows were receiving a good grade of alfalfa hay and an increased amount of grain. The vitamin G content of the milk followed rather closely the vitamin G content of the grasses, although the vitamin G content of the

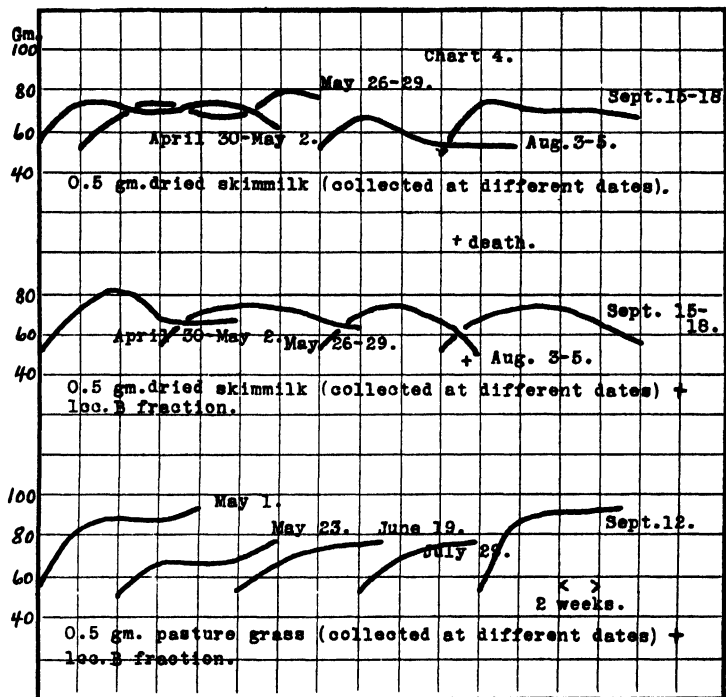


CHART 4. Vitamin B content of samples of milk and grass

milk is somewhat lower than that of the grass (weight for weight on air-dried basis). What is true in an extreme drought year is no doubt true to a less extent every year (results of first experiment in this paper) for the results as stated above were no doubt partially due to the stage of maturity at which the samples of grasses were harvested. As a matter of record the moisture, protein, and ash content of these samples of grasses are recorded in Table I.

The vitamin B content of the same samples of milk and grass is

represented by the curves shown in Chart 4. Vitamin B is the limiting factor for growth in milk and grass. The B fraction from yeast extract accentuates this fact, for we find a different curve in the case of milk when we compare the curves for milk alone with those for milk supplemented with the B fraction. Milk collected on August 3 to 5 showed a slightly lower vitamin B content in that all of the rats receiving milk and the B fraction supplement died of polyneuritis. The early summer and fall pasture grasses appear to have a higher vitamin B content than the midsummer grasses. These observed facts may, however, depend upon the temperature and the amount of moisture in the soil, indicating that vitamin G in grasses, or hay made from these grasses, is synthesized during the process of rapid growth, and then is dissipated as the plant matures. On this basis it would seem that the highest quality of hay would be produced by cutting it while the plant is still immature. This appears to be in keeping with practical observations and feeding results.

SUMMARY AND CONCLUSIONS

Milk from cows on pasture has a higher vitamin G content than milk from cows on dry feed, although the quality of the hay used in dry feeding may be a determining factor. The vitamin B content of the milk is not so affected.

Cows on early pasture during its vigorous state of growth produce milk higher in vitamin G than do cows on an overmature pasture. While the vitamin B content is not so easily affected, there are slight indications that fresh tender grass produces a milk higher in vitamin B than does overmature pasture grass.

It appears that vitamin G is synthesized during the process of rapid growth, and then is dissipated as the plant matures.

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THE LACK OF RELATIONSHIP BETWEEN THE CALCIUM, PROTEIN, AND INORGANIC PHOSPHORUS OF THE SERUM OF NON-NEPHRITIC CHILDREN

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It has been shown repeatedly (1-4) that in cardiac and renal disease, both in adults and children, there is a direct relationship between the levels of serum protein and serum calcium, and also, within limits, an inverse relationship between the serum inorganic phosphorus and the serum calcium (1-5). Peters and Eiserson (4) attempted a mathematical evaluation of the relative effects of protein and phosphorus on serum calcium, intended to permit the prediction of the calcium concentration of the serum in so far as affected by the levels of serum protein and phosphorus. It was assumed that close correspondence between observed and calculated values indicated the absence of direct disturbance of calcium metabolism. More recently, Peters and Van Slyke (6) point out that "the equation (4) must be considered as only a rough first approximation of the effects of phosphate and protein on serum calcium." The absence of direct relationship between serum protein and serum calcium has, however, been used as a criterion for distinguishing primary disturbances of calcium metabolism (7).

It is obvious that the interrelationships of protein, calcium, and inorganic phosphorus in the sera of infants and children cannot be identical with those of adults, for in children, the inorganic phosphorus of the serum is higher, and the protein lower, than in the serum of adults; both of these changes, according to theory, tend to decrease the level of the serum calcium, whereas the serum calcium of the children is usually higher than that of adults. It may be argued that growth itself constitutes a primary alteration in the calcium metabolism, and therefore changes in serum calcium

coincident with changes in serum protein or phosphorus are not to be expected. Such changes do occur, however, in renal disturbance in children (1-3), yet these children may not lose their power to grow.

If the protein and inorganic phosphorus of the serum exert a definite influence upon the level of the serum calcium in nephritic children, it is necessary to determine whether these factors are of major importance in determining the level of the serum calcium in healthy children. There are fairly well marked changes in the levels of serum protein and inorganic phosphorus between birth and maturity. The serum protein of new born infants is low, and rises rather rapidly through infancy and early childhood (8). The serum inorganic phosphorus reaches a maximum value at about 4 to 6 months of age, and decreases gradually throughout childhood (9). If either protein or inorganic phosphorus exerts a definite influence, *per se*, upon the level of serum calcium in normal infants and children, it should be readily demonstrable by comparing the levels of serum calcium at different ages throughout childhood.

A series of determinations of calcium, total protein, and inorganic phosphorus in 76 sera from children ranging in age from birth to 16 years has been obtained. The infants were all well, but the group of older children included some with various diseases not considered as affecting the calcium metabolism nor involving the renal function. The distribution of the cases in childhood is given in Table I.

Blood was taken with as little stasis as possible from the arm or jugular vein in the older children, from the longitudinal sinus of the infants, and from the cord vein of the new-born. The blood was centrifuged immediately, and the serum separated from the clot within 15 to 30 minutes after collection. Hemolyzed sera were rejected. Serum calcium was determined by the method of Kramer and Tisdall (10), 24 hours being allowed for complete precipitation of the calcium oxalate; the method of Fiske and Subbarow (11) was used for phosphorus; and total protein was determined by micro-Kjeldahl analysis (12), the determined non-protein nitrogen being subtracted from the total nitrogen.

All of the analyses were made during the summer months. The mothers of the new born infants had been given cod liver oil for at

TABLE I
Classification of Cases

Classification	No. of observations
No disease, weight normal, new born infants	18
" " " " infants, 1-10 mos.	16
" " " " children, 1-16 yrs.	6
Children 5 to 20 per cent underweight	7
" with diseases of musculature*	10
" " mental deficiency; hysteria	8
" " unoperated orthopedic deformities†.	5
Miscellaneous group (diseases of upper respiratory system, syphilis,† bladder exstrophy)	6
Total	76

* Poliomyelitis rehabilitation cases, dermatomyositis.

† No cases of bone syphilis, bone tuberculosis, or active rickets included.

‡ Not including bone syphilis.

TABLE II
Serum Calcium, Inorganic Phosphorus, and Total Protein of Infants

New born infants			Well infants 1-10 mos.			
Calcium	Inorganic P	Protein	Age	Calcium	Inorganic P	Protein
mg. per 100 cc.	mg. per 100 cc.	gm. per 100 cc.	mos.	mg. per 100 cc.	mg. per 100 cc.	gm. per 100 cc.
10.6	4.6	4.8	1	11.2	7.4	6.7
10.8	5.2	6.8	1	10.3	6.6	4.4
10.8	6.2	5.3	1½	10.6	5.6	5.6
11.2	4.8	5.7	1½	10.8	7.5	5.3
11.2	6.0	5.9	1½	10.9	4.3	4.9
11.1	5.9	5.2	2	10.6	6.5	5.7
11.6	4.7	6.2	3	11.5	7.0	5.5
11.6	6.4	6.4	3½	12.1	7.0	6.4
11.7	5.0	6.0	4	10.8	6.8	6.0
11.7	5.5	6.6	4	11.7	6.5	5.1
11.7	6.1	6.0	4½	11.7	6.6	6.7
11.7	6.7	6.1	5	11.6	6.7	6.1
11.8	4.6	5.9	5	11.5	6.6	5.6
11.8	6.1	5.6	6	12.0	6.7	5.7
12.0	6.3	6.5	8½	11.2	6.3	6.2
12.1	6.5	5.2	9½	11.4	5.6	6.6
12.5	5.4	5.3				
12.5	7.0	5.9				

least a month before parturition; the infants and children were given cod liver oil daily and were out of doors during the major part of the day. The serum calcium and phosphorus values were thus at a maximum (13-16). The inorganic phosphorus values of the infants are definitely higher than the values usually reported

TABLE III

Serum Calcium, Inorganic Phosphorus, and Total Protein of Children 1 to 16 Years of Age

Age	Calcium	Inorganic P	Protein	Age	Calcium	Inorganic P	Protein
<i>yrs.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>yrs.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>gm. per 100 cc.</i>
1½	11.9	6.7	6.2	11	10.8	4.1	7.6
2	10.2	5.2	7.3	11	11.6	6.2	4.4
2	11.5	4.8	6.4	11	10.8	4.7	6.7
2	11.1	5.6	7.6	11	11.0	4.6	7.3
2	11.0	5.4	7.9	11	11.7	5.7	8.1
3	11.5	4.8	6.7	12	11.1	5.7	7.1
3	10.1	6.5	7.2	12	11.3	4.2	8.0
3	10.9	5.0	7.5	12	11.2	4.4	8.1
5	11.5	6.4	7.6	12	10.0	4.2	5.6
8	11.9	4.3	7.3	13	11.9	3.9	7.9
8	10.5	5.1	5.8	13	12.1	3.4	5.8
8	10.9	4.7	7.8	13	10.7	4.6	7.5
9	11.3	4.8	7.7	13	11.0	3.8	7.3
9	12.0	3.9	6.5	13	10.8	4.7	6.8
9	12.0	5.5	7.4	13	10.4	4.6	7.4
9	12.1	5.5	7.1	13	10.1	5.3	7.3
9	12.4	5.5	7.3	14	11.6	4.6	7.1
10	12.5	5.8	8.6	14	11.4	4.0	7.6
10	11.4	5.5	7.1	14	11.6	3.5	7.5
10	10.9	4.7	7.5	15	10.9	3.8	6.9
10	10.9	4.6	4.1	15	11.5	3.7	6.8
10	11.3	5.4	7.5	16	12.0	3.8	6.4
11	12.0	6.1	7.6				

as normal. These values were all obtained from well infants in our experimental ward, whose intake of milk approximated 1 quart daily, who were given cod liver oil, and in addition, were out of doors several hours a day. In this locality the amount of sunlight is high and the smoke nuisance practically nil during the summer months. These high values for serum inorganic phos-

phorus have been observed during two successive summers, with two groups of well infants, and have therefore been considered as physiological, rather than pathological elevations.

The complete data are given in Tables II and III. In Table IV the data are grouped, first according to inorganic phosphorus

TABLE IV

Non-Variation of Serum Calcium of Children with Physiological Changes in Serum Protein

No. of deter- minations	Protein		Inorganic P, average	Calcium	
	Range	Average		Range	Average
3 to 4 mg.					
	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
5	5.8-6.9	6.5	3.7	10.5-12.0	11.7
3	7.3-7.9	7.6	3.7	11.0-11.9	11.5
4 to 5 mg.					
6	4.1-5.9	5.2	4.5	10.0-11.8	10.9
6	6.2-7.1	6.6	4.7	10.8-12.2	11.4
11	7.3-8.1	7.6	4.5	10.4-11.9	11.1
P = 5 to 6 mg.					
4	4.7-5.8	5.3	5.5	10.5-12.5	11.2
6	6.0-7.1	6.7	5.4	10.8-11.7	11.3
10	7.3-8.1	7.6	5.4	10.1-12.4	11.3
P = 6 to 7.5 mg.					
9	4.4-5.6	5.2	6.6	10.3-12.5	11.3
10	5.7-6.2	6.0	6.6	10.6-12.5	11.5
7	6.4-7.6	6.9	6.5	10.1-12.1	11.6

content then within the "constant" phosphorus levels, according to increasing protein content. Table V shows the same data grouped according to increasing phosphorus content, within "constant protein" levels. It is evident from these tables that serum calcium shows no constant variation with changes in either the serum protein or the inorganic phosphorus levels. In fact, the

TABLE V

Non-Variation of Serum Calcium of Children with Physiological Changes in Serum Inorganic Phosphorus

No. of determinations	Protein, average	Inorganic P, average	Ca, average
	<i>gm. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
7	5.2	4.5	11.1
5	5.3	5.5	11.2
13	5.4	6.6	11.4
6	6.5	3.4	11.5
8	6.7	4.7	11.5
7	6.6	5.4	11.3
10	6.3	6.6	11.6
3	7.6	3.7	11.5
12	7.6	4.5	11.5
10	7.6	5.4	11.3
3	7.5	6.3	11.2

TABLE VI

Serum Calcium, Protein, and Inorganic Phosphorus of Nephritic Children

Reference	Calcium	Inorganic P	Protein	Reference	Calcium	Inorganic P	Protein
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>gm. per 100 cc.</i>		<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>gm. per 100 cc.</i>
Blackfan and Hamilton (2)	6.6	4.7	2.8	Boyd, Courtney, and MacLachlan (3)	7.2	6.3	5.0
	6.8	5.4	2.6		8.8	6.0	5.0
	7.6	6.8	3.8		9.0	9.0	6.5
	8.0	6.5	4.3		9.2	5.5	6.0
	8.2	4.3	4.7		9.4	3.8	6.1
Salvesen and Linder (1)	9.0	4.0	7.8	Authors' data	9.8	3.0	6.5
	6.3	9.0	4.87		6.1	9.5	6.1
	6.9	6.4	4.66		6.6	4.4	4.8
	7.3	5.0	4.35		8.6	6.6	4.4
	7.5	5.3	3.77		9.4	2.4	5.5
	7.6	5.8	4.34		9.4	3.5	6.3
	7.7	8.0	4.52		9.5	3.9	5.5
	7.8	6.7	4.37		10.1	4.5	8.3
	8.1	5.6	4.68		12.6	4.7	7.2
	8.1	7.3	5.15				
	8.2	6.3	4.59				
	8.4	2.9	5.95				
	8.5	6.7	4.52				

TABLE VII

Variations of Serum Calcium of Nephritic Children with Changes in Serum Protein

No. of deter- minations	Protein		Inorganic P, average	Calcium	
	Range	Average		Range	Average
P = 2.4 to 4 mg.					
	<i>gm. per 100 cc.</i>	<i>gm. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
3	5.5-5.9	5.6	3.1	8.4-9.5	9.1
3	6.1-6.5	6.3	3.4	9.4-9.8	9.6
P = 4 to 5 mg.					
3	2.8-4.8	4.1	4.5	6.6-8.2	7.1
4	7.2-8.3	7.8	4.3	9.0-12.6	10.5
P = 5 to 6 mg.					
2	2.6-4.0	3.2	5.3	6.8-7.5	7.1
3	4.3-4.7	4.4	5.5	7.4-8.1	7.7
1		6.0	5.5		9.2
P = 6 to 7 mg.					
4	3.8-4.5	4.2	6.6	7.6-8.6	8.0
5	4.5-5.0	4.8	6.3	6.9-8.5	7.9
P = 7.0 to 9.5 mg.					
2	4.5-5.0	4.7	8.5	6.3-7.7	7.0
3	5.2-6.6	6.0	8.6	6.1-9.0	7.7

TABLE VIII

Variations of Serum Calcium of Nephritic Children with Changes in Serum Inorganic Phosphorus

No. of determinations	Protein, average	Inorganic P, average	Ca, average
	<i>gm. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
3	4.1	4.5	7.1
3	4.4	5.5	7.7
9	4.5	6.4	7.9
2	4.7	8.5	7.0
3	6.3	3.4	9.6
1	6.0	5.5	9.2
3	6.0	8.6	7.7

data bring out rather strongly the striking constancy of the serum calcium level throughout infancy and childhood.

For purposes of comparison, a series of thirty-three analyses of sera of nephritic children has been collected from the literature (1-3) and from our own data. These values are given in Table VI, and the averages, computed in the same manner as in Tables IV and V, are shown in Tables VII and VIII. It will be observed (Table VII) that, even though the series is small, there is a definite increase in serum calcium with increase in serum protein. When the inorganic phosphorus is 6 mg. or over, the phosphorus level apparently becomes an active factor in the regulation of the serum calcium of nephritic children, as is to be expected (5).

DISCUSSION

It is of interest that physiological changes in the serum protein are without apparent effect upon the level of the serum calcium in childhood, while pathological changes in this constituent exert a marked effect, even though the extent of the change in the latter case may be no more marked than in the former. Likewise, physiological increases in serum inorganic phosphorus above 6 mg. per cent are not accompanied by a decrease in serum calcium, as is observed with pathological increases in phosphate. These facts become particularly noteworthy when one realizes that 85 per cent of the experimental data from which the formula (4) expressing the relationship between the calcium, inorganic phosphorus, and protein in the serum of adults was derived, were obtained from patients with renal or cardiac disease.

That serum protein is a major factor in determining the level of the serum calcium is a most attractive theory from the physico-chemical standpoint (17). There is no question but that there is a parallelism between the serum protein and calcium levels in nephritis. Not only the level of the serum calcium (1-4) but also the concentration of calcium in other body fluids in nephritis varies directly with the protein content of these fluids (1). Experimental evidence of the comparative protein and calcium contents of different body fluids in non-nephritic animals tends also to show a parallelism between protein and calcium levels (7, 18). On the other hand, in disturbances of the secretion of the parathyroids the level of the serum protein is apparently without

effect upon the level of the serum calcium (19). The parathyroid hormone undoubtedly influences the level of the serum calcium in normal individuals. The relative importance of the several factors influencing the amount of calcium in the serum in normal individuals has not yet been experimentally proved, for the differing results obtained with sera of normal and nephritic children must cast doubt upon the validity of using data from nephritic patients as criteria for judging non-nephritics.

The studies so far reported on the relation of serum protein and calcium in non-nephritic adult patients are not wholly convincing. The decrease in both serum calcium and protein observed during late pregnancy has been quoted in support of the above theory. The relationship noted here is more apparent than real, however, for Oberst and Plass (20), in a series of simultaneous determinations of calcium and protein in the serum of pregnant women, found that the serum protein decreases during the early months of pregnancy, while the serum calcium remains at nearly the normal level during early pregnancy, decreasing during the later stages of gestation. After parturition, the serum protein rises more rapidly than the serum calcium. If the decrease of serum calcium during pregnancy is dependent upon the decreased protein level, it is difficult to understand why there should be such a marked lag in the fall of serum calcium during pregnancy, when there is apparently no such lag in nephritis.

Merritt and Bauer (7), in a study of calcium and protein content of serum in patients with mental disease, found that in a series of thirty-four patients with non-suppurative diseases of the central nervous system there was apparently a direct relationship between the serum calcium and the serum protein. Their conclusions were drawn from a study of averages. In five patients whose serum protein was between 5 and 6 per cent, the serum calcium varied from 8.40 to 9.57 mg. per cent; in twenty-one patients with serum protein of from 6 to 7 per cent, the serum calcium range was 8.40 to 10.50 mg. per cent; and in seven patients with serum protein values of from 7.0 to 8.43 per cent, the serum calcium range was 9.48 to 10.14 mg. per cent.

In a group of twenty-one patients with meningitis these authors concluded that there was no correspondence between the serum protein and calcium which fact they considered as indicative of

primary disturbance of calcium metabolism in meningitis. In this second series there were four patients with serum protein between 5 and 6 per cent, the calcium variation observed was from 7.8 to 10.2 mg. per cent; in fifteen patients with a serum protein between 6 and 7 per cent, calcium values of from 8.40 to 9.70 mg. per cent were obtained; and the two patients whose protein values were above 7 per cent showed 9.45 and 9.36 mg. per cent serum calcium. The range of serum calcium values in the two groups brings out rather clearly the possible error in drawing conclusions from averages of a small number of values covering a rather wide range. The differences in range of serum calcium per gm. difference in serum protein are not conspicuously different in the two groups, and are by no means as sharply marked as was observed in the series of nephritic children (Table VII), also a small group.

From the marked differences observed between the sera of nephritic and non-nephritic children, and from the paucity of data from non-nephritic adults in the literature, it would seem that more experimental data are needed in order to determine the exact influence of serum protein and serum phosphorus on the serum calcium of non-nephritic adults.

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SUMMARY

A series of 76 sera from infants and children varying in age from birth to 16 years has been analyzed for calcium, inorganic phosphorus, and total protein. No data from nephritic or cardiac patients, nor from patients with obvious disturbances of the calcium metabolism were included in the series.

No constant relationship was observed between the level of serum calcium and serum protein or serum inorganic phosphorus.

These findings are in direct contrast to the findings in nephritis in childhood.

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EFFECTS OF DEPRIVATION OF MANGANESE IN THE RAT*

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Manganese is indispensable for normal plant growth (1). Its indispensability in animal nutrition has hitherto not been demonstrated, although a considerable literature exists dealing with the content of manganese in various parts of the bodies of animals, and with a description of the effects of adding manganese salts to animal rations poor in this element.¹ All ordinary foodstuffs contain manganese in small amounts, and the content of the element in animal tissues is much lower than in vegetable foods. It is not surprising, therefore, that no results of a conclusive nature have been recorded to establish whether or not manganese is an indispensable element in mammalian nutrition, or to reveal the specific effects of manganese starvation. The present study was planned to secure this information.

The objective of our study was the preparation of a diet complete in every respect, but as nearly as possible free from manganese. For estimating the manganese content of the food we had the choice between the periodate method, and the spectrographic examination of the ash of the constituents of the diet. The periodate method will not give reliable values in the presence of organic matter or chlorides. The spectrographic method is much more rapid and convenient than is the chemical method, and affords unequivocal qualitative information as to the presence or absence

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¹ A bibliography covering the content of manganese in foods, various organs and tissues, and experimental work on the significance of manganese in nutrition to September, 1930, is supplied by Richards (Richards, M.B., *Biochem. J.*, **24**, 1573 (1930)).

of the elements. This method enabled us to detect manganese in certain foods which, on the basis of chemical tests, have been reported as manganese-free.

The spectrographic method has, however, two disadvantages. It is not quantitative to a high degree and quantitative only when very small amounts of manganese are present. It likewise requires expensive equipment. Quantitative results by the spectrographic technique are subject to errors of at least 10 per cent and sometimes 25 per cent. Differences in concentrations can only be demonstrated with certainty by the spectrographic method when they are of a magnitude of about 50 per cent. For example, actual concentrations of 1.0 and 1.25 p.p.m. can probably not be distinguished by the spectrograph, but 1.0 and 1.5 can be with certainty. Since our objective was solely to be certain that the manganese content of the food employed was extremely low, the limitations of the analytical procedure were of no importance, and our technique had the advantage of reliability from a qualitative standpoint.

Technique of Spectrographic Analysis of Foodstuffs for Manganese

With progressive dilution of an element in a spectral source there is a corresponding diminution in the spectral lines of that element. This diminution manifests itself both in the number of lines and in their intensities. Those lines which persist longest as the element is progressively diluted are the *raies ultimes* of de Gramont (2). He stated that the *raies ultimes* of manganese are 2576.2, 2593.7, and 2605.7. Line 2576.2 is the first *raie ultime* because a smaller concentration of the element is required for its excitation than is required for any other. As the concentration or quantity of manganese in the spectral source is increased the next *raie ultime* to make its appearance is 2593.7. It is the second *raie ultime*. Line 2605.7 is the third *raie ultime*, since it is the third line to appear as the concentration or amount of manganese in the spectral source is increased. In this study only that part of the spectrum was examined in which the *raies ultimes* of manganese may be found. The spectrograph used in this investigation is the Hilger E₁ quartz prism spectrograph, of the "Littrow" type. It disperses the spectrum from 1930 to 8000 Å. over a distance of 30 inches and is, therefore, suitable for the study of complex spectra.

This 30 inch spectrum is photographed in 10 inch sections on 10 × 4 inch photographic plates. Only the desired region in the ultra-violet which would show the *raies ultimes* of manganese, viz. 2576.2, 2593.7, and 2605.7, was photographed.

A Hartmann diaphragm and a spherocylindrical quartz lens are accessories to the equipment. The electrodes used were copper rods 5 mm. in diameter made for this purpose and supplied by Hilger. They were of high purity and showed few lines other than those due to copper, and manganese lines were absent. The electrodes were supported in a Hilger high tension spark holder. This is equipped with a separate rack and pinion mechanism so that each electrode can be independently raised or lowered. The holder itself is equipped with another rack and pinion mechanism by means of which both electrodes can be raised or lowered together simultaneously without disturbing the spark gap between them or their position with respect to each other.

The spectrum was excited by a condensed spark which was produced by a hook up consisting of a 20,000 volt step-up transformer, ratio 1:200, having a 0.02 m. fd. condenser across its secondary circuit and a self-induction coil in series with the secondary circuit, and a variable resistance in the primary circuit. The resistance in the primary circuit was used for reducing the primary voltage from 118 to 90 volts, which was found optimal for the transformer. The self-induction served to smooth the spark discharge and reduce the intensities of the "air lines" which otherwise would be present in the straight condensed spark.

Eastman No. 36 photographic plates were found satisfactory. An elon-hydroquinone developer was employed. This type is especially effective for contrast work. The fixer used was ordinary Eastman acid hypo.

Details of the Spectrographic Technique

The details of the technique were as follows: A pair of the copper electrodes is prepared by pointing one of them and drilling into the end of the other a hole about 4 mm. in diameter and 3 mm. deep. These electrodes are fitted into the Hilger spark holder, the pointed electrode being used as the upper, and the one containing the hole as the lower electrode. They are then adjusted with respect to one another so that the point of the upper is 7 or 8 mm.

directly above the hole in the lower electrode. The holder is then brought into a position about 30 cm. in front of the slit of the spectrograph. The electrodes are next connected with the spark, which is first turned on as a source of light in locating or placing the spark laterally and vertically with reference to the slit. Before so doing the prism of the spectrograph is rotated into a position in which it directs a visible portion of the spectrum into the field to be covered by the plate. The spark is then turned on and moved vertically and laterally until its image appears in the center of the prism field on looking at the prism from a point within the scope field to be covered with the plate. The spherocylindrical lens is then interposed between the slit and the spark and adjusted until a sharp image of the spark is focused on the slit. This operation completes the adjustment of the position of the spark with reference to the slit. The prism may then be rotated and focused for the spectral region containing the manganese *raies ultimes*, viz. 2576.2, 2593.7, and 2605.7.

When this adjustment has been made, the plate holder containing the plate is inserted into its place and racked into position for photographing a spectrogram, preferably or usually on the uppermost portion of the plate. Then the dark slide is withdrawn in order to expose the plate to the light from the spark. A "spectrogram" will consist of three spectra in juxtaposition to one another. They are made possible by the Hartmann diaphragm by means of which three successive segments or portions of the slit can be exposed separately for the photographing of the three successive spectra. The *uppermost* of these spectra is that of the empty copper electrodes. It is photographed by maintaining the spark across the empty electrodes for the required length of time which, in the case of the plates used, was 60 seconds, and by keeping exposed at the same time the *lower* portion of the slit through the lower aperture in the Hartmann diaphragm. This first is a control spectrum which demonstrates whether or not the electrodes contain the element to be tested for, in this case manganese. The lower electrode is then detached and its hole plugged full of the ash or other material to be examined and then returned to its position in the holder. After sliding the middle aperture in the Hartmann diaphragm over the slit so as to expose its central portion, the spark is turned on again for the same length of time, viz. 60

seconds. By this exposure the second spectrum is photographed. This second spectrum is that of the unknown substance, that is, the substance which is being examined, in our study for manganese. The third spectrum is a contrast spectrum for placing or locating the manganese lines in the middle spectrum. It is photographed in the same manner as the second except that a small amount of manganese is added to the contents of the hole in the lower electrode. It will, therefore, contain manganese lines which will locate any manganese lines contained in the middle spectrum or second spectrum. In photographing the third spectrum, the uppermost portion of the slit is exposed by the corresponding aperture of the Hartmann diaphragm. It will be noticed that the successive portions of the slit used in photographing the above three spectra are in the reverse order in which the spectra appear on the plate. This reverse order is due to inversion of the images by the optical system of the spectrograph.

In order to use the spectrographic method as a technique for estimating manganese quantitatively in biological ashes of unknown manganese contents, it was first necessary to ascertain or establish the exact correlations which will exist between known concentrations of manganese in biological ashes and the manganese *raies ultimes* in the spectra of such ashes when these spectra have been prepared by the same spectrographic technique which will subsequently be applied to biological ashes of unknown manganese content. That is, it was first necessary to determine the concentrations of manganese which will produce in standard spectrograms the several varieties and numbers of manganese lines which occur in the spectrograms of biological ashes whose manganese contents are unknown and are to be determined by the manganese lines which they produce. Or, it was necessary to determine and identify the manganese lines which known concentrations of manganese will produce. The manganese contents of such unknowns can then be assumed to be equal to the particular concentrations of manganese previously found to be required to produce the same manganese lines in standard spectrograms as those observed in the spectrograms of the unknowns. In order to establish these correlations, standard spectrograms were prepared by the above described technique of a series of manganese standards consisting of artificial ashes containing such a range of manganese concentrations

as were expected to occur in biological ashes. The artificial ashes were a mixture of equal parts of c.p. K_2HPO_4 and $CaH_4(PO_4)_2$ which was assumed to act in the spark as a natural biological ash. This artificial mixture was used in place of a natural ash since there was greater assurance of complete absence of manganese from such a c.p. mixture than from any natural ash. The manganese standards were prepared by adding and mixing into 100 gm. portions of the above artificial ash mixture the required quantities of manganese in the form of solutions containing 0.01 per cent to 0.1 per cent of the element manganese as a chloride, and then drying the resulting mixture at a temperature of about 110° . The different concentrations prepared and $MnCl_2$ solutions used in their preparations are listed in Table I.

TABLE I
Concentration of Manganese Standards with Artificial Ashes

Mn solution per 10 gm. ash	Per cent on basis of ash	P.p.m. on basis of fresh tissue when ash represents 1 per cent of fresh substance
<i>cc.</i>		
0.0	0.000	0.0
1.0 (0.01 per cent)	0.001	0.1
5.0 (0.01 " ")	0.005	0.5
1 0 (0.10 " ")	0.010	1.0
2.0 (0.10 " ")	0.020	2.0
5.0 (0.10 " ")	0.050	5.0
10.0 (0.10 " ")	0.100	10.0

The values in the third column of Table I are the p.p.m. which the several corresponding concentrations of manganese in the ash represent of a fresh tissue of which the ash is 1 per cent.

Sensitivity

Table II was prepared from the results of an examination of the spectrograms. It shows that 0.1 p.p.m. on the basis of fresh tissue can be detected by this spectrographic technique. The absence of manganese line 2576.2 is evidence of the absence of manganese down to less than 0.1 p.p.m. in fresh tissue. A manganese concentration of approximately 0.1 p.p.m. is demonstrated by the presence of line 2576.2 in the absence of line 2593.7. The

presence of lines 2576.2 and 2593.7 is evidence of a manganese concentration of 0.5 p.p.m. The presence of all three manganese lines indicates a manganese concentration of 1.0 p.p.m. or more.

Preparation of Sample

In preparing biological material for examination, the technique of ashing the samples as described by McCollum, Rask, and Becker (3) was followed. In ascertaining the absence or presence of an element so widely distributed as manganese, precautions to pre-

TABLE II

Quantitative Spectrographic Analysis of Manganese Standards with Artificial Ashes

Mn, p.p.m. on basis of fresh tissue	Wave-lengths in International Ångström units		
	2576.2 μ_1	2593.7 μ	2805.7*
0.0			
0.1	±		
0.5	+	±	
1.0	++	+	±
2.0	+++	++	+
5.0	+++	+++	++
10.0	+++	+++	+++

de Gramont (2) designates the ultimate rays by the symbols μ_1 , μ_2 , μ_3 ; μ_1 being the last to disappear. The letter μ without the subscript indicates that the order of disappearance has not been established with certainty.

± indicates when seen by lens only; +, faintly but clearly visible to the eye; ++, distinctly visible to the eye; +++, much more intense.

* Next sensitive ray.

vent contamination must be exercised in preparing the sample. All dishes and instruments were carefully cleaned as for quantitative analysis. Care was taken when obtaining a representative sample that it at the same time remain in its natural condition. In vegetable matter, for instance, in the case of the potato, a cube was cut out from the center of it and used as the sample. The usual precautions were also taken in obtaining the various organs of the rat for analysis. After the animal was killed, a ventral mid-line incision was made, the body was carefully skinned in such a manner as to prevent any contact with hair and foreign material or from touching the exposed parts. The material to be examined was placed in previously prepared silica dishes in

which it was ashed immediately by placing the dishes on silica triangles supported over Bunsen flames. After the sample was ashed, the silica dishes were covered, placed underneath small bell jars, and stored in this manner until examined spectrographically as already detailed.

TABLE III
Spectrographic Analysis of Manganese Content of Plant Substances

Substance	Wave-lengths in International Ångström units		
	2576.2	2593.7	2605.7
Potato.....	+++	+++	++
Yeast	+++	+++	++
Harris yeast concentrate.....	+	±	±?
Dextrin.....	+	+	±
Agar-agar.....	+++	+++	++
Starch (corn, Argo).....	±?		
Wheat germ.....	+++	+++	+++
Lettuce.....	+++	+++	+++
Tomato.....	++	+	+
Vioosterol	±?		
Peanuts.....	+++	+++	+++
Almonds.....	++	+	±
Walnuts.....	+++	++	+
Lima beans.....	++	+	+
Soy beans.....	++	++	+
Sucrose.....			
Wheat germ oil.....	+	±	±?
Corn.....	+	±	±
Chlorophyll.....			
Whole grapefruit.....	+	+	±
Grapefruit juice.....	+	±	±?
Whole orange	++	+	+
Orange juice.....	+	±	±
Filter paper clippings.....	±		

This technique was applied to all plant and animal products examined for their manganese content. The principal classes of human foods were analyzed for their manganese content. Tables III and IV list the plant and animal products examined in their natural state.

The data contained in Tables III and IV can be regarded as a sufficient survey of biological materials for their manganese con-

tents. They show that the manganese contents of plant tissues range from less than 0.5 p.p.m. to 10 p.p.m. and higher, and that the manganese contents of animal tissues range from 0.1 p.p.m. to 5 p.p.m. These values agree in general with those of previous

TABLE IV
Spectrographic Analysis of Manganese Content of Animal Substances

Substance	Wave-lengths in International Ångström units		
	2576.2	2593.7	2605.7
Egg (whole).....	+	+	
Alcohol-egg yolk extract.....	+		
Egg albumin.....	+?		
Whole milk.....	+		
Casein (crude).....	+?		
“ (prepared in this laboratory).....			
Dry skim milk (Merrell-Soule)....	+?		
Butter fat.....			
Halibut.....	+?		
Trout.....	+?		
Salmon.....	+	+?	
Cod.....	+	+	±?
Bluefish.....	+	+?	
Salmon liver.....	+++	++	+
Cod liver oil.....	+?		
Lard.....	+?		
Beef muscle.....	±		
“ spleen.....	±	±?	±?
Hog “.....	±	±?	±?
Bone (beef).....	±	±	±?
Urine (human).....	±	±?	±?
Carious teeth (human).....	±	±?	
Beef liver.....	+++	+	+
“ kidney.....	+	±	±?
“ lung.....	±	±?	±?
“ heart.....	±?	±?	

investigators. However, there are a few interesting and probably significant differences between the data in Tables III and IV and those of previous investigators. For instance, these data show that the several species of fish muscle contain about 0.1 p.p.m. to 0.5 p.p.m. of manganese, whereas Lindow and Peterson

(4) found the same muscle of the same species of fish to be manganese-free.

The data in Tables III and IV show that an adequate diet will invariably contain manganese if prepared from natural foodstuffs. Accordingly, man and other animals have never subsisted on diets free from manganese, a fact of special interest and significance in this study.

In the preparation of an adequate but manganese-free diet there are available according to the data contained in Tables III to V, starch and sucrose as carbohydrates, casein as a protein, butter fat as a source of fats, and vitamins A, D, and E, and the following mineral salts: NaCl, K_2HPO_4 , NaH_2PO_4 , $CaH_4(PO_4)_2$, and calcium lactate. However, the data in these tables also show that for an adequate diet the following essential dietary constituents are not available in a manganese-free condition: supplementary proteins, a source of vitamins B and G, an iron salt, and a magnesium salt. The preparation of these dietary essentials in a manganese-free condition constituted the next step in this study.

Preparation of a 50 Per Cent Alcoholic Yeast Extract

Since dilute alcoholic extracts of yeast contain vitamins B and G, a 50 per cent alcoholic yeast extract and a 70 per cent alcoholic yeast extract were prepared and examined for their manganese content. Both of these extracts were manganese-free. A 50 per cent alcoholic extract of yeast was used in the manganese-free diet as a source of these vitamins and also as a source of proteins to supplement casein. The details of preparing this extract are as follows:

To 500 gm. of a high grade of dry yeast powder² contained in a 5 liter Pyrex balloon flask were added 5 liters of cold 50 per cent alcohol by volume. The contents were shaken at 15 minute intervals for a period of 3 hours and then allowed to stand overnight at room temperature. On the following morning the extract was decanted through acid-washed filter paper and then transferred immediately to the refrigerator, where it was stored until mixed into the diet.

² Prepared by the Northwestern Yeast Company, Chicago.

As will be described in a succeeding section, this extract was incorporated in the diet without previous evaporation or concentration. The alcohol and water were evaporated after the ex-

TABLE V
Spectrographic Analysis of Manganese Content of Various Chemicals

Chemical	Wave-lengths in International Ångström units		
	2576.2	2593.7	2805.7
HCl.....	±	±?	
H ₂ SO ₄	±	±?	
NaOH.....	±?		
FeCl ₂	+	+	±?
NaCl.....			
NaH ₂ PO ₄ + H ₂ O.....			
CaH ₄ (PO ₄) ₂			
Ca lactate.....			
CaCl ₂			
K ₂ HPO ₄			
Fe citrate (ic).....	+	+	±?
MgSO ₄	+	±	
MgO.....	±	±	
MgCl ₂	+	±	±?
Mg citrate.....	±	±?	±?
“ salicylate.....	±	±	±?
“ lactate.....	±		
Ferric ammonium sulfate, Fe ₂ -(SO ₄) ₃ ·(NH ₄) ₂ SO ₄ ·24H ₂ O.....	++	+	+
Electrolytic iron (Bureau of Standards).....	+	+	±?
Fe ₂ O ₃ (Brandt).....	+	+	±?
Fe wire (pure).....	++	++	+
FePO ₄ ·4H ₂ O.....	++	++	±
Fe ₂ (SO ₄) ₃ ·9H ₂ O.....	++	+	+
Fe(OH)(C ₂ H ₃ O ₂) ₂	+	±	±
Copper oxide.....	±?		
“ acetate.....	±	±	±?
“ carbonate.....	±?		
“ chloride.....	+	+	±
“ nitrate.....	±?		
“ sulfate.....			
“ sulfide.....	++	+	+
Distilled water.....			
Tap water.....	±	±	

tract had been incorporated with the other dietary constituents by spreading the diet mixture in layers about 1 inch thick in Pyrex biscuit trays, and exposing it to a current of air at 45–50°. The preparation of the extract was scheduled so that no extract was more than 3 days old before incorporation with the diet.

Preparation of Manganese-Free Iron

Table V shows that all of the iron salts examined for manganese contained it. The iron preparations tested were the best obtainable and were purchased on specification that they be manganese-free.

After many unsuccessful attempts to prepare iron free from manganese it was found that this could be done by a modification of the procedure of Moore and Miller (5). The iron is precipitated from the ferric chloride solution by pyridine instead of NH_4OH . The details of this modified procedure are as follows:

Dissolve 25 gm. of FeCl_3 in 25 cc. of 4 N HCl . To the solution add a few drops of concentrated HNO_3 and heat to 50–60° to oxidize the iron. Dilute to 250 cc. and add a sufficient quantity of redistilled pyridine³ to precipitate all of the iron. Allow the iron hydroxide to settle and decant the supernatant liquid through a filter. Transfer the precipitate to the filter and wash with pyridine water (1:500) until the washings contain no colloidal iron. The iron hydroxide is redissolved in 4 N HCl , and reprecipitated by pyridine. The process is repeated eight times. This ferric hydroxide is dried at 45–50°. It contained less than 0.5 p.p.m. of manganese.

Preparation of Manganese-Free Magnesium

All magnesium salts and other sources of magnesium examined contained at least traces of manganese. As shown in Table V the magnesium preparations examined for manganese included all of the ordinary salts which had been selected from c. p. labeled bottles and preparations supplied on specification that they be manganese-free. Accordingly, the preparation of manganese-free magnesium presented itself. Our own efforts showed that all known methods of purifying magnesium salts were ineffective

³ Merck's Medicinal grade.

for removing such traces of manganese as could be detected. The idea was then conceived of removing the manganese by first converting it into permanganate by means of KIO_4 .

Attempts to free magnesium from manganese by precipitation as $\text{Mg}(\text{OH})_2$ from a solution of magnesium sulfate after oxidation of the manganese to permanganate with KIO_4 in acid solution were unsuccessful. Accordingly, the manganese must be removed before precipitation as hydroxide. Electrolysis of a magnesium sulfate solution after conversion of the manganese to permanganate by KIO_4 was finally accomplished in a special apparatus devised to prevent convection currents in the system. This method is described elsewhere (6). By this procedure an abundance of magnesium hydroxide spectrographically free from manganese was easily prepared.

EXPERIMENTAL

Four experiments were carried out. In the first, the diet was manganese-free. The second was the control diet made up of the same substances as the manganese-free ration but without the manganese having been removed. The third diet was the manganese-free ration plus 0.005 per cent of manganese as $\text{MnCl}_2 + 4\text{H}_2\text{O}$, which approximates the average amount of manganese in plant and animal tissues. The fourth ration consisted of the manganese-free diet with the addition of 0.05 per cent of manganese. The composition of these diets and the salt mixtures used in them are given below.

Composition of Diets⁴

Diet 1 (Manganese-Free)

	per cent
50 per cent alcoholic yeast extract—100 cc.; = yeast.....	10.0
Casein.....	20.0
Salt Mixture 185 (modified).....	4.0
Butter fat.....	8.0
Corn-starch.....	58.0
Vioosterol, 15 drops per kilo.	

⁴ The rats received iodine in water once a week and distilled water daily. Both of these were found to be spectrographically manganese-free.

Effect of Mn Deprivation

Diet 2 (0.005 Per Cent Manganese)

	per cent
50 per cent alcoholic yeast extract—100 cc.; = yeast.....	10.0
Casein.....	20.0
Salt Mixture 185 (modified).....	4.0
Butter fat.....	8.0
Corn-starch.....	57.982
MnCl ₂ ·4H ₂ O.....	0.018
Viosterol, 15 drops per kilo.	

Diet 7 (0.05 Per Cent Manganese)

	per cent
Same as Diet 2 except that the content of MnCl ₂ ·4H ₂ O is	0.18
And therefore the corn-starch.....	57.82

Diet 3 (Control)

	per cent
Yeast.....	10.0
Casein.....	20.0
Salt Mixture 185.....	4.0
Butter fat.....	8.0
Dextrin.....	58.0
Viosterol, 15 drops per kilo.	

*Composition of Salt Mixture 185**

	mg.
NaCl.....	173.0
MgSO ₄ (anhydrous).....	266.0
NaH ₂ PO ₄ +H ₂ O.....	347.0
K ₂ HPO ₄	954.0
CaH ₄ (PO ₄) ₂ +H ₂ O.....	540.0
Fe citrate (ic).....	118.0
Ca lactate.....	1300.0

* Before use the salt mixture was treated with water in the proportion of 30 gm. of the fresh powder to 15 cc. of distilled H₂O and heated at 100° for 30 to 45 minutes in a constant temperature oven.

Composition of Salt Mixture 185 (Modified)

	mg.
NaCl.....	173.0
Mg(OH) ₂ *.....	128.8
NaH ₂ PO ₄ +H ₂ O.....	347.0
K ₂ HPO ₄	954.0
CaH ₄ (PO ₄) ₂ +H ₂ O.....	540.0
Fe(OH) ₃ *.....	100.0
Ca lactate.....	1300.0

* Prepared free from manganese.

The diets were made up in kilo lots. All the utensils were glass or porcelain. The components were well mixed, then the yeast extract was added, and the wet mixture was spread in Pyrex biscuit trays which were exposed to a current of air at 45–50° to evaporate the alcohol and water. The ration was then ground in a glass mortar and placed in glass jars. These were kept in the refrigerator to prevent the butter fat from becoming rancid.

Throughout the experimental period, spectrograms were prepared of the ashes of these diets, thus constantly checking the manganese content of the rations prepared at different times. Table VI shows the manganese content of these diets according to spectrographic analysis.

TABLE VI
Spectrographic Analysis of Experimental Diets

Diet	Wave-lengths in International Ångström units		
	2576.2	2593.7	2605.7
Manganese-free.....			
Control.....	+	+	+
Diet containing 0.005 per cent Mn.	+++	+++	+
“ “ 0.05 “ “ “	+++	+++	+++

Observations on Rats Fed a Manganese-Free Diet

Growth—Young rats were raised to maturity on diets containing no manganese, 0.005 per cent of manganese, and 0.05 per cent of manganese. The rats so raised were compared as respects growth, estrous cycle, blood regeneration, fertility, success in rearing young, and general well being over a considerable portion of the normal span of life, with control rats raised under identical conditions on a diet consisting of the same substances from which the manganese had not been separated, but otherwise identical with the manganese-free diets. The composition of the control diet and the test diets is tabulated in Diets 1, 2, 3, and 7. Three separate series of experiments were carried out.

The rats used were born of our breeding stock and weighed 40 to 50 gm. each when started on the diets. They were placed in groups of six in cages provided with screen bottoms. When the females had become pregnant they were isolated in individual

cages in which Grade A filter paper clippings, washed in hydrochloric and hydrofluoric acids, and which spectrographic tests showed to be practically free from manganese, were used for bedding. Porcelain bowls were used for feeding. The rats were weighed weekly and hemoglobin determinations and erythrocyte counts were made once a month.

The growth curves of the rats fed the manganese-free diet did not differ from those of well nourished rats in our colony. Weights of 250 to 350 gm. were not unusual in animals which had been

TABLE VII
Manganese Content of Experimental Animals

Sample	Wave-lengths in International Ångström units								
	2576.2			2593.7			2605.7		
	Mn-free diet	Control diet	Diet containing 0.005 per cent Mn	Mn-free diet	Control diet	Diet containing 0.005 per cent Mn	Mn-free diet	Control diet	Diet containing 0.005 per cent Mn
Pancreas.....		++	++		+	+			+
Testes.....		+	+		+	+			+
Kidney.....	±?	+	++	±?	+	+	+		+
Ovary.....		+	+			+			+
Spleen.....		+	+		±?	+			+
Liver.....		++	++		+	++	+		+
Heart.....		+	+		±	+			?
Lung.....		+	+		±?	+			
New-born.....	±?	+	+		+	+		+	+
15 day old rats.....	±?	+	+			+			+

7 or more months on the diet. Their growth and appearance did not differ noticeably from those groups receiving 0.005 per cent, those receiving 0.05 per cent respectively of manganese, or from the controls. Some of the young of each group were examined spectrographically for the presence of manganese. These examinations were applied to the ashes of the entire bodies of new born rats, of the entire bodies of rats 15 days old, at which time they had had no food other than the mother's milk, and to the ashes of individual organs of the first generation of progeny of rats on

these diets after they had reached maturity and had subsisted on the respective experimental diets for about 8 months. The organs examined included the liver, kidney, spleen, pancreas, heart, lung, testes, and ovary. The manganese contents of these ashes of entire rats and of organs are recorded in Table VII.

These data show that the organs of rats on the manganese-free diet were practically free from manganese. Both spleen and liver showed none of the *raies ultimes* of manganese, thus indicating its absence. The spectrum of kidney showed a doubtful line, hardly visible under a lens, which signifies a manganese content of less than 0.1 p.p.m. The organs of the rats on the other test diets, as well as the control, showed a manganese content of 5 to 10 p.p.m.

New born rats and rats 15 days old of mothers on the manganese-free diet appear to contain no manganese, whereas those from mothers on the other test diets do show the presence of this element. This indicates that manganese is transmitted through the placenta.

Blood Regeneration—The rats on the manganese-free diet were observed for possible effects of lack of manganese on blood regeneration. Hemoglobin determinations and erythrocyte counts were made once each month. The results, as shown in Table VIII, fell within the normal limits and compared very favorably with those of rats on the other two test diets supplemented with manganese (0.005 and 0.05 per cent of manganese respectively), as well as with those of the controls. These observations indicate that absence of manganese from the diet and from the body does not disturb the process of formation of hemoglobin and erythrocytes. Table VIII gives the details of blood examinations on seven rats on the manganese-free diet. The data on the other groups on experimental diets are not presented because they represent normal conditions.

Anemia was induced in young rats by restricting them to a diet of pasteurized and certified whole milk, and in other experiments by restricting rats to a diet of powdered skim milk (Merrell-Soule). The latter induced the desired degree of anemia sooner than the liquid milk diet. There was no loss in weight but rather a slow steady gain, and the time required to induce anemia of the degree desired was somewhat longer than was expected from the experience of some other workers. The feeding of manganese- and

TABLE VIII
Hemoglobin Determinations and Erythrocyte Counts of Rats on Manganese-Free Diet

Date	Rat 1		Rat 2		Rat 3		Rat 4		Rat 5		Rat 6		Rat 7	
	R.b.c.*	Hb†	R.b.c.	Hb	R.b.c.	Hb	R.b.c.	Hb	R.b.c.	Hb	R.b.c.	Hb	R.b.c.	Hb
<i>1929</i>														
Sept. 25.....	8.04	12.92	5.34	12.78	6.91	12.25	6.27	10.02	6.27	13.45	6.29	10.65	8.20	12.65
Oct. 25.....	8.94	13.05	8.64	13.72	8.36	14.51	7.66	10.42	8.44	12.92	7.56	13.98	9.53	14.65
Nov. 25.....	8.49	13.98	8.19	13.98	9.70	14.11	8.49	15.31	8.68	13.98	9.34	13.98	9.43	13.98
Dec. 25.....	7.82	13.18	9.26	13.32	8.81	12.52	7.58	12.92	7.47	13.45	8.08	12.78	9.05	13.72
<i>1930</i>														
Jan. 25.....	7.30	13.72	7.76	12.92	7.60	14.38	7.65	13.05	7.50	12.65	7.55	13.58	7.71	13.05
Feb. 25.....	7.60	13.32	8.00	13.72	7.55	13.58	7.76	12.92	7.74	13.58	7.60	14.38	7.87	13.98
Mar. 25.....	7.49	14.25	7.96	14.78	7.86	13.85	7.98	15.06	8.04	13.45	8.86	15.31	8.08	14.11

* The red blood cell count is taken per c.mm.

† Measured in gm. per 100 cc.

copper-free iron did not enable the rats to recover from their anemia. Likewise the feeding of manganese (free from copper) together with iron, did not enable rats to increase the hemoglobin content. Our results, therefore, support the indispensable relation between the copper and iron content of the diet for the synthesis of hematin.

Estrous Cycle—Evans and Bishop (7) have reported studies on the relation between diet and fertility. Their method of observing the exterior changes in the vagina, the vaginal smear, contents of the vaginal lumen, the diestrous interval (five stages), was followed with the manganese-free and manganese-fed rats, the examinations being made 6 days per week and at about the same time every day. These observations showed that the cycle in the manganese-free rats was similar and compared well with those of the control animals and with those receiving the diets to which manganese was added.

Notes on Rats Fed Diet 1 (Manganese-Free)

The total number of rats fed the manganese-free diet (Diet 1) was 131. Of these 72 were males and 59 females. In the first experiment 60 rats, thirty males and thirty females, were used. They were restricted to the diet during 14 months. There were no deaths in this group during the school year of 1929-30, but in the summer of 1930 five rats died due to excessively hot weather. That this was the cause of death was made certain by the death of thirty-one of our stock rats during an excessively hot night. All these rats were in fine condition up to the time of death, and those which survived the period of hot weather remained normal in external appearance. The following notes illustrate the reproductive histories of the females restricted to Diet 1.

Reproduction Histories of Females

Group 1

Rat 1—After 3½ months on the diet it had four young born dead. These were the progeny of manganese-free males whose sperm cells were probably defective. At the time of mating the male had been on the diet 94 days. 3 weeks after the delivery of the first litter this rat was mated with a stock male. A litter of seven young was produced, of which one died within 24 hours. A second litter of nine was later produced, of which two were dead. Of the twenty young born of this mother thirteen were reared. These were

kept on the manganese-free diet for 8 months. There were five males, which were not examined until the age of 120 days. They showed no spermatazoa but some spermatids. These males were distinctly undersized, never reaching a weight of more than about two-thirds the normal expectation based on the growth of males on Diet 1.

The eight females which were progeny of Rat 1 were reared on the manganese-free diet. They were undersized, had rough coats and scaly tails, and were highly irritable. When mated with stock males each bore one litter, after which all but one was killed. This one produced a second litter, which was neglected.

The history of Rat 1 differed markedly from that of any other fed Diet 1, as will be seen from the typical histories of several other females in the group, which follow.

Rat 2—After 115 days on Diet 1 this rat bore a litter of two young, both dead, probably because of defective sperm cells. 3 weeks later she was mated with a stock male. Eventually she produced three litters, one of eight young, four of which were still-born. The remaining ones died within 24 hours. A second litter of ten was all dead within 3 days from neglect. A third litter contained six, four of which were born dead. The remaining ones died the next day from neglect. None of the young of this and other rats of Group 1 had milk in their stomachs except those of Rat 1.

Rat 3—This rat had no young while with males reared on Diet 1 to 100 days of age. After mating with a stock male she produced two litters, one of eight, of which one was still-born. Two died within 3 days, and the remaining ones within a week from lack of nourishment. In the second litter of six, two were still-born, the others died within 24 hours from neglect.

The histories of the remaining females in Group 1 are so similar to those of Rats 2 and 3 that they are not presented.

Group 2

Rat 1—At about 100 days of age this rat bore a litter of eight, all of which died within 2 days without having suckled. Their stomachs were empty. These were all the progeny of stock males.

Rat 5 had a litter of six, all dead when first seen. Another litter of eight all died within 2 days from neglect. There was no milk in the stomachs of any of these young.

These histories are typical of those of the other females of Group 2, and the others are not presented.

The second group consisted of 71 rats, of which twenty-nine were females. These have been on the diet 9 months. Certain of the females were killed at intervals for histological study. All had delivered young, which they had failed to nurse. Except among the young there was no mortality in the group.

The average number of litters per rat thus far secured was

three. The average number of young per litter was seven. The mortality was 100 per cent. Thus, but one female in 59 reared young and she succeeded with but thirteen of twenty, all of which were inferior in size and appearance.

*Experiments with a Diet Made Manganese-Free to Which
Manganese Was Added*

Twenty females were raised on a diet identical with Diet 1 except that manganese was added in the form of the chloride to the extent of 0.005 per cent (manganese) of the food mixture. Young were successfully weaned in almost all litters. The females did not differ noticeably from stock rats in their attitude toward caring for their young. For example, four rats in one experiment were kept until each had two litters, a total of 67 young. Three of a litter of nine were apparently still-born. The remaining 64 were weaned and appeared normal.

Four of the 64 progeny described in the preceding paragraph were raised on the manganese-free diet and were kept until two had produced one litter each, and the others two litters each. One lost her litter of six within 2 days. Another weaned two of a litter of six. A third weaned all of two litters of seven young each. The fourth weaned eleven of fourteen born in two litters. It appears that this low content of manganese is near the minimum amount which enables the rat to react normally toward her young and produce adequate milk for their nourishment.

Nineteen females were raised on Diet 7, which was like Diet 1 except that 0.05 per cent of manganese was added in the form of the chloride. One rat weaned all of two litters of five and six respectively. A second weaned thirteen of twenty born in three litters. A third weaned sixteen of twenty-one in three litters. A fourth weaned all of her first litter of eight, and five of a litter of ten, of which five were still-born. A fifth weaned all of a litter of seven. These were typical of the entire group.

Of these young two females were restricted to the diet on which their mothers had been raised. They appeared normal. When mated with males of the same group one weaned all of a litter of nine, and the other all of a litter of seven, but lost six of another litter of nine.

Three females and three males of the third generation just

described were continued on Diet 7 and mated. One weaned all of her first litter of eight but lost her second litter of twelve. A second reared twenty-three of twenty-five young in three litters. The third reared all of a litter of seven. There was no apparent inferiority in any of these young of the fourth generation on Diet 7. These results show that the addition of manganese to Diet 1 rendered it complete for the support of reproduction and suckling of young.

Experiments on Exchanging Young of Normal and Manganese-Free Mothers

Tests planned to show whether normal stock females would accept the young of manganese-free mothers and nurse them were made. For years it has been our custom to allow stock mothers to nurse not more than eight young, to prevent underfeeding. Each day the new litters are examined and young in excess of eight are given to mothers with smaller litters. In some thousands of transfers from one mother to another we have no record of one refusing foster young. The raising of manganese-free young by stock mothers was tried to show whether the young were normal and whether failure of manganese-free females to rear young was due to defect in the young or in the mother.

A total of 107 young of mothers in Group 2 (Diet 1) were given to stock mothers to nurse. Of this number but one rat was weaned. It was retarded in growth, although it was the only one with the mother and therefore had an abundance of milk. The stock mothers abandoned or ate the manganese-free young. In several tests manganese-free young were wrapped in cotton with normal young from 1 to 3 hours, in order to transfer the odor of one to the other. In these cases the stock mother nursed the normal young successfully, but the manganese-free young, except as noted, did not secure milk. In a number of other tests where manganese-free young, with or without previous contact with normal young, were given to stock mothers, none was reared except in the single instance mentioned.

We are not yet prepared to decide whether the death of the young of females fed the manganese-free diet was due to lack of initiative (except in the few which survived) in seeking to suck, or to indifference of the mother in giving them an opportunity. The

latter explanation is best in accord with our observations on the mother's behavior. Almost all failed to form a nest, collect their young, or to hover over them. In several cases we observed the birth of the young and saw the other litters when they were from an hour to a few hours old. The mothers appeared indifferent to their young. Normally nourished females show strong maternal instincts and their care in forming a nest and in hovering over their new born young, and their resentment at interference with them are well known.

The most successful female in this group in rearing foster young was one which had been 6 months on the manganese-free diet before being mated with a stock male. She delivered a litter of seven which appeared to be in excellent condition and were normal in weight. She had failed in former litters to suckle her young and after the birth of the litter mentioned she was deprived of her own young which were transferred to a stock female which had just delivered a litter. The young of the stock mother were given to the manganese-free rat. The latter had constructed a nest for her young when they were first seen. When given the stock young she formed a nest but at first did not remain with them to permit them to nurse. She made too many excursions around the cage, leaving the young in the nest restless from hunger. They were watched but were not seen to nurse during the 1st day. The next morning one was observed holding a nipple. Later another was nursing. The young seemed hungry and aggressive. The mother appeared to submit at times to the efforts of the young to suck, rather than to encourage them and take satisfaction in their contact with her and with the suckling act.

On the 8th day all seven were alive, but during the 2nd week the mother destroyed five. The remaining two weighed 45 and 51 gm. when weaned at 30 days of age, showing that they had received enough milk. Litters of two and three grow much faster than do larger litters. Even a low milk production would have accounted for the growth of these young.

Another female in this group delivered five young, one of which was dead. The young were left with the mother for 3 hours, at which time there was no milk in their stomachs. The rat did not show any interest in the young and had not formed a nest. They were then transferred to a stock female which had just delivered

a litter, that was given to the manganese-free mother. The latter at first was indifferent to the young and gave no attention when they were handled in her presence. At the end of the day the young had not sucked. The next day the young were extremely hungry and were making vigorous efforts to get at the female to suck, but she remained away and gave them no chance. At about noon one succeeded in securing hold of a nipple, and about an hour later another had attached itself and was sucking. 3 days later all the young were being nursed, the attitude of the female having changed. She now snatched up a young rat when disturbed and hurried away with it for its protection, and would industriously gather them together when they were scattered about the cage. During the 2nd week two young disappeared from the cage, doubtless having been eaten by the foster-mother. The remaining five young were weaned in good condition at weights of 34 to 40 gm.

The stock female which had exchanged young with the manganese-free female, although an excellent mother, declined to nurse the manganese-free young. The latter were all dead within 3 days.

With the few exceptions noted, the females, although fertile, had no success in nursing their young. The newly born manganese-free young were of normal weight and appearance but showed no milk in their stomachs.

We shall report at another time the results of histological studies on several tissues of the manganese-free rats. It may be noted at this time that even in the two instances where females suckled their young and reared some of them, the young were markedly undersized although the number was small and the milk yield necessary for their normal growth was distinctly less than the yield normally produced by females nursing litters of eight. This fact supports the view that the mammary tissue is not adequately developed in these females. Their lack of solicitude for encouraging their young to nurse points to the same conclusion.

Since, as will be described later, the germinal epithelium in the male rat on the manganese-free diet degenerates completely, it seems not improbable that there may be a tendency to imperfection in the ova derived from females suffering from manganese deficiency. This is made probable by the observation that an unusual number of young were born dead, or died in a period too short for starvation. We are not certain whether any of the

young were still-born, for some were delivered during the night and were not seen for several hours. Whether this explanation be true or not, it is certain that the detrimental effect, if any, of manganese starvation on female rats is limited to failure or deficiency of milk secretion, and to lack of maternal solicitude in caring for the young.

Effects of Manganese Deprivation on Male Rats

Male rats grow normally on Diet 1 and show no abnormalities until about 90 to 100 days on the diet. They then become sterile



FIG. 1. The upper specimens are testes of rats 115 days on the manganese-free diet + 0.005 per cent manganese, the lower testes of rats 105 days on the manganese-free diet.

owing to immotility of their sperm cells. After about 150 days on the diet many males show no sperm cells but many spermatids. During the period of degeneration of the germinal epithelium the testes decrease in size. They may show sperm cells even after they have decreased to about half the normal size (Fig. 1).

72 male rats were raised on Diet 1, all of which showed testicular

degeneration. After $9\frac{1}{2}$ months on the diet the testes were about the size of those of a 6 weeks old rat. They were soft, flabby, cyanotic, translucent in appearance, edematous, and had undergone tubular destruction. The atrophy rapidly progresses. The epididymis finally degenerates completely until only vestiges remain. Sterility was demonstrated in these rats both by testicular smears and by mating tests. By 90 to 100 days they became indifferent to females which were placed in the cage, whereas normal males always examine them with interest. There is little or no obesity in these males.

We have not yet completed our studies on the extent to which the degeneration of germinal epithelium and other structures in the testes can proceed and yet be checked by manganese feeding, nor how far regeneration may be brought about at different stages in the degeneration process by this means.

As controls we raised a series of eleven male rats on Diet 2, containing 0.005 per cent of manganese, and fifteen males on Diet 7, containing 0.05 per cent of this element. We also examined four males raised on another control diet made of the same substances as Diet 1 but without removal of the manganese. These males were all tested and found by mating experiments to be potent after intervals of 9 to 14 months on the control diets. After these periods on the diets they were examined by smears and found to be producing an abundance of motile spermatozoa. On each diet males were raised to the fourth generation and were shown to be potent.

Histological studies of the testes and of various other organs and tissues of the rats on all of the diets employed in this study are in progress. The testicular atrophy and the failure of females to suckle young suggest failure of some hormone production in the hypophysis. There is a clinical literature on male sterility developing in middle life which is referred to hypophyseal deficiency. There is likewise recognized by endocrinologists a stimulating effect on milk secretion caused by the administration of certain hypophyseal extracts. Observations thus far made seem best explained on the theory that manganese is in some manner related to hormone formation by the hypophysis. The problem is being studied in detail.

SUMMARY

1. A method is described for the preparation of a diet essentially free from manganese.

2. On the manganese-free diet young rats grow to maturity in an apparently normal manner. The females, as shown by the technique of Evans and Long, involving daily examination of vaginal smears, go through normal estrual cycles. They produce approximately the normal number of young if mated at proper intervals with normal males. These females, deprived of manganese, failed in 58 of 59 cases to suckle their young. They appeared indifferent to them and did not give them the care or opportunity to suck which is characteristic of female rats on the same dietary formula with small amounts of manganese added.

These manganese-free females, when given foster young from our stock litters failed in eight cases out of ten to suckle them, although the vigorous young were very aggressive because of hunger. The seven young reared by the other two females were retarded, apparently from insufficient milk supply.

Female rats of our breeding stock did not show interest in and therefore did not give an opportunity for the young of manganese-free mothers to suck. But seven of 107 manganese-free young were reared by stock foster-mothers. These were undersized and of inferior appearance although the litters were small and in all likelihood secured all the milk they wanted.

3. Male rats raised on a manganese-free diet showed no abnormality other than testicular degeneration. This degeneration is well under way by the 100th day on the diet. The atrophy then rapidly progresses until only vestiges remain and complete sterility results. There is little or no obesity in these males.

The same manganese-free diet, after the addition of 0.005 to 0.05 per cent of manganese in the form of the chloride kept males in sexual potency for at least 14 months. Their testes appeared normal at the end of the experiment.

4. Manganese is absent (spectrographic test) in the young of manganese-free mothers. It is present in new born and 15 day old rats of mothers on manganese-containing diets. Manganese therefore passes through the placenta if present in the mother's blood. It is also absent from the organs of rats raised on the experimental diet.

5. Manganese is shown to have no rôle in blood regeneration.

6. As a working hypothesis, based on similarity of clinical evidence of human subjects with the observed effects of manganese deficiency on rats of both sexes, we suggest the provisional hypothesis that manganese is in some manner concerned with the production of a hormone by the anterior lobe of the hypophysis, which is essential for the functioning of the testes and for the proper development and functioning of the mammary tissue.

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STUDIES IN KETONE BODY EXCRETION

I. DAILY VARIATIONS IN THE KETONE BODIES OF NORMAL URINE AND THE KETONURIA OF SHORT FASTS, WITH A NOTE ON DIABETIC KETONURIA DURING INSULIN TREATMENT

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By means of a technique, to be described later in this paper, which permits the determination of the ketone bodies in such concentrations as are found in normal urine, a study has been made of the total excretion of these substances by normal individuals and of the variations which take place in this excretion during various periods of the day. The increase in the amount of ketone bodies excreted by a normal individual in the early stages of fasting has also been investigated and preliminary studies have been made of the ketonuria of diabetics under insulin treatment.

1. Normal Excretion of Ketone Bodies

Definite figures for the amount of the ketone bodies eliminated by normal individuals are not numerous in the literature. According to Peters and Van Slyke (1), the total acetone body excretion, expressed as β -hydroxybutyric acid, is usually less than 0.5 gm. in 24 hours and probably rarely exceeds 1 gm. Shaffer (2) considered any amount over 0.1 gm. of total acetone per day as distinctly abnormal. Hubbard and Noback (3) found a variation of from 0.00 to 3.8 mg. per 100 cc. of urine in the normal excretion of total acetone, the acetone and diacetic acid varying from 0.00 to 1.2 mg. with an average of 0.19 mg., and the β -hydroxybutyric acid (as acetone) from 0.00 to 2.6 mg., averaging 0.46 mg. per 100 cc. of urine. For general purposes urine samples which give negative reactions with nitroprusside have been considered normal with regard to acetone and diacetic acid. As has been pointed out

elsewhere (4), the degree of acetonuria necessary to give a positive nitroprusside test varies considerably with variations in the method and in the ratio of acetone to diacetic acid present. It is probably equivalent to not less than between 3 and 5 mg. of diacetic acid per 100 cc. of urine.

The present experiments have covered the acetone and diacetic acid excreted by twelve normal individuals during the 24 hours, the results representing thirty-two daily periods. Determinations were also made of the β -hydroxybutyric acid excretion of eight individuals during fourteen daily periods. The results may be summarized as follows:

Ketone Bodies Excreted in 24 Hours (as Acetone)

From acetone and diacetic acid.	1.2 to 4.5 mg.,	average	2.9 mg.
" β -hydroxybutyric acid ..	12.2 " 20.5 "	" "	16.2 "
" total acetone bodies.....	14.5 " 23.5 "	" "	19.4 "

Ketone Bodies per 100 Cc. Urine in 24 Hour Samples (as Acetone)

From acetone and diacetic acid.	0.10 to 0.66 mg.,	average	0.25 mg.
" β -hydroxybutyric acid...	0.79 " 2.20 "	" "	1.30 "
" total acetone bodies.....	0.91 " 2.70 "	" "	1.50 "

A study of large numbers of unselected urine samples (such as come into this laboratory for routine analysis) by means of a sensitive qualitative test for acetone and diacetic acid (4) also indicates that the normal concentration of the urinary acetone and diacetic acid is below 0.5 mg. per 100 cc. of urine.

2. Variations in Normal Ketone Body Excretion Throughout the Day

Almost nothing is to be found in the literature regarding the variation in normal ketone body excretion during the 24 hours, although there have been reports on the daily variations under dietary conditions which produced marked ketonuria. The daily curves for ketonuria under the different conditions which have been reported are not identical, but in each case the variations during the day seem to bear a relation to certain specific factors. The highest excretion followed ketogenic meals and periods of exercise (Hubbard and Wright (5), McClellan and Toscani (6)) and the lowest excretion occurred at night (Hubbard and Wright) or in the morning (McQuarrie and Keith (7), McClellan and Toscani). A summation effect from two meals, or a lag in the appearance of

ketone bodies, was sometimes noted (Hubbard and Wright, McClellan and Toscani). Hubbard and Wright also present one series of figures for an arthritic subject on a normal diet, which shows almost no variation throughout the day, but they believe these figures to be below the limits of accuracy of the method.

We studied the daily variations in urinary output of ketone bodies of ten normal individuals, the observations covering thirty-six daily periods. Each voiding was collected and analyzed separately. For comparative purposes we have grouped the figures for the separate voidings into four periods for each day, as shown in Table I. As nearly as possible the morning period represents in each case the time between waking and taking lunch, the afternoon period, the time between lunch and dinner, the evening period, from dinner to bed time.

The subjects were all apparently normal individuals, under normal conditions.¹ The figures for sugar in all of the samples (with the exception of one sample) were normal. Seven of the subjects were women, three men.

Six typical examples of these 24 hour periods are shown in Table I, and the range of variation and average figures for all of the results of each period are also shown.

The variations in ketone body excretion during the day are very slight in most cases. There is, however, a distinct tendency for the amount of ketone bodies excreted per hour to be lower at night than during the day, while the percentage concentration tends to be lower at night than during the afternoon and evening, but lowest of all during the morning.

When the variations in hourly excretion of ketone bodies are compared with those of urinary volume for the same periods, it is found that the two curves almost always vary in the same direction, though not to the same degree. This is somewhat more noticeable in the β -hydroxybutyric acid than in the acetone-diabetic acid fraction, although in general the two ketone body fractions vary in the same direction. The relationship between hourly

¹ One of the subjects was a possible exception, having suffered several years earlier from a nephritic condition from which there had been an apparent recovery. No abnormalities were found in the urine of this individual except that one sample, excreted just after lunch, showed 0.5 per cent sugar.

Ketone Body Excretion. I

TABLE I
Showing Ketone Bodies and Urinary Volume Excreted by Normal Individuals during 24 Hours
 The ketone bodies are expressed in terms of acetone.

Experiment No.	Morning						Afternoon						Evening						Night					
	Acetone and diacetic acid			β-Hydroxy-butyric acid			Acetone and diacetic acid			β-Hydroxy-butyric acid			Acetone and diacetic acid			β-Hydroxy-butyric acid			Acetone and diacetic acid			β-Hydroxy-butyric acid		
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	cc.	Vol-ume of urine per hr.
1	0.06	0.09	0.50	0.75	150	0.16	0.08	1.30	0.66	51	0.16	0.08	1.30	0.66	51	0.12	0.05	1.00	0.46	46				
2	0.16	0.18	0.70	0.80	115	0.15	0.11	1.25	0.90	72	0.42	0.09	2.64	0.60	23	0.20	0.04	1.77	0.41	23				
3	0.12	0.11	0.90	0.81	90	0.14	0.13	1.39	1.28	92	0.24	0.10	1.65	0.71	43	0.20	0.06	1.53	0.45	30				
4	0.22	0.08	2.10	0.76	36	0.16	0.12	1.20	0.92	77	0.19	0.07	1.65	0.58	35	0.11	0.03	1.50	0.39	26				
5	0.13	0.14	1.04	1.09	105	0.10	0.13	0.76	1.00	132	0.20	0.12	1.83	1.06	58	0.20	0.11	1.40	0.76	54				
6	0.20	0.15	0.92	0.73	77	0.48	0.19	1.60	0.65	41	0.32	0.15	1.50	0.69	46	0.27	0.09	1.53	0.49	32				
Average figures for thirty-six daily periods of ten individuals.....																								
Highest.....	0.15	0.14	1.04	0.97	94	0.28	0.15	1.44	0.78	54	0.26	0.12	1.69	0.81	48	0.21	0.06	1.58	0.50	32				
Lowest.....	0.52	0.19	2.10	1.30	253	0.80	0.36	2.08	1.28	132	0.80	0.31	2.64	1.12	144	0.40	0.16	3.16	0.76	68				
	0.06	0.04	0.35	0.41	22	0.10	0.03	0.76	0.56	11	0.10	0.04	0.93	0.30	23	0.08	0.02	0.18	0.25	12				

excretion of ketone bodies and urinary volume is particularly striking when shorter periods than those shown in Table I are observed. Chart 1 illustrates one of the many cases in which a large volume of urine excreted during a short period is paralleled by a great increase in ketone body excretion, unaccounted for by other conditions.

This correlation between hourly excretion of ketone bodies and urinary volume, independent of the time of day, was further shown by the experiment illustrated in Chart 2.

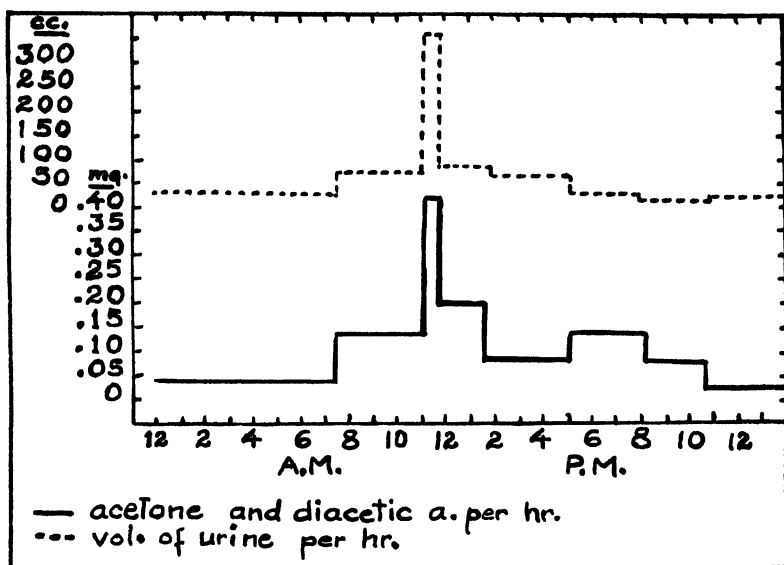


CHART 1

The subject of this experiment usually drank coffee and water for breakfast, which resulted in a marked diuresis during the morning. Much less liquid was usually taken at night. The first period shown in Chart 2 illustrates one of the characteristic daily curves for this individual, with a low hourly volume of urine and low acetone figures during the night, and an increase in both during the morning. In the second period the usual custom was reversed and the subject drank large amounts of water at 9.30

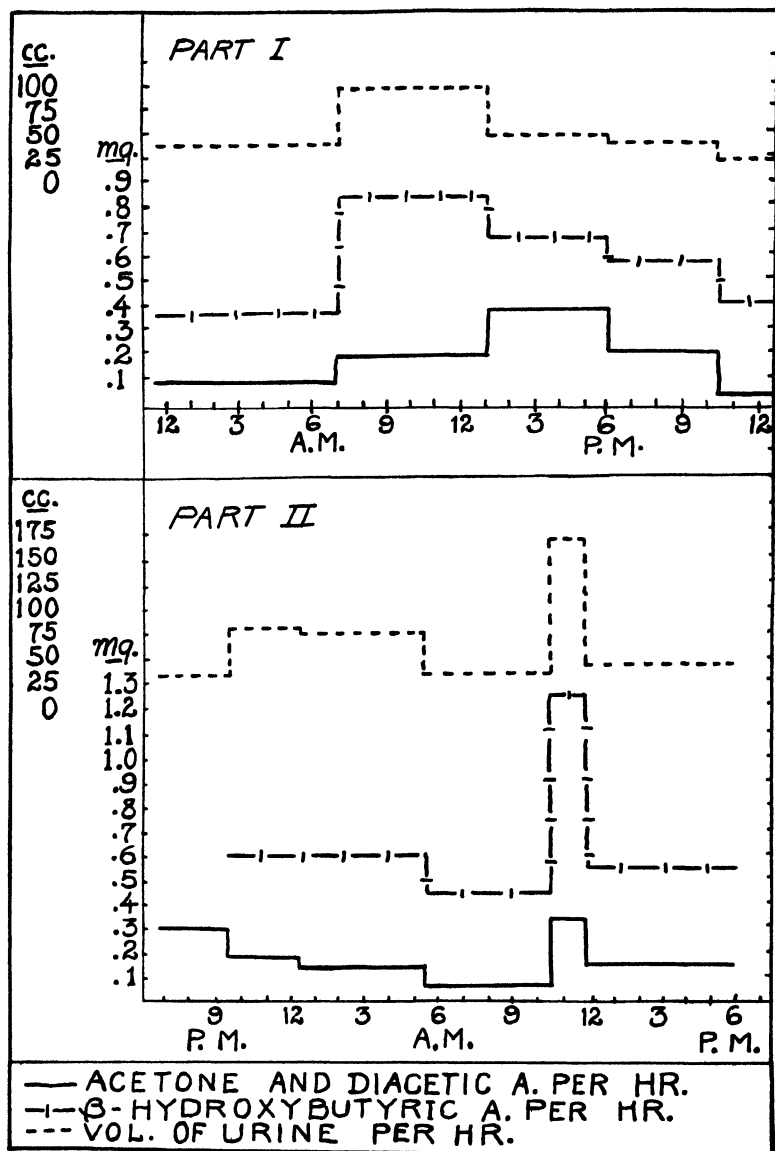


CHART 2

p.m. and again at 12.30 a.m., and following this took almost no liquid for breakfast. The result was an increase in urinary volume during the night, accompanied by a greater excretion of ketone bodies than usual during this period. From 5.30 a.m. when the subject rose, to 10.30 a.m., there was a decrease in ketone bodies excreted, parallel with the decrease in urinary volume. At 10.30 a.m. 300 cc. of water were taken, which resulted in a diuresis and increase in ketone body excretion similar to the condition which usually occurred during the first period of the morning.

It is evident that the factor of urinary volume should be taken into account in any consideration of variations in the amount of ketone bodies excreted. An apparent lag in excretion may be due to the washing out, by large volumes of fluid, of ketone bodies formed during an earlier period.

That urinary volume is not the only factor determining variations in the normal excretion of these bodies is apparent from the fact that the percentage concentration also varies.

In so far as the actual amount of a substance excreted per unit time varies in a direct relation to urinary volume, the implication is that the substance is simply washed out by the kidneys, and that variations in the actual amount excreted are not in themselves significant of the time or the conditions of formation of the substance. From this point of view variations in percentage figures may have a particular significance as representing changes in the amount of the substance washed out per unit volume. More extensive studies of all urinary constituents on this basis would be of interest.

In their study of the ketonuria produced in arthritic subjects by ketogenic diets, Hubbard and Wright (5) found the rate of excretion of ketone bodies to be independent of urinary volume, and the figures of McClellan and Toscani indicate that the same is true of the ketonuria of men on meat diets. It is possible that this is indicative of a difference in mechanism involved in normal and in increased excretion of ketone bodies.

To summarize, our figures indicate that in the normal subject the highest excretion of ketone bodies occurs during the afternoon and evening, that during the night there is a decrease both in percentage and in absolute amount excreted, and that during the morning there is a still greater decrease in percentage concentration

although the actual amount excreted per hour is greater than at night, the increase accompanying the increase in urinary volume, and representing, perhaps, a washing out of substances formed during the night. These variations are, in general, in accord with those which have been noted in the ketonuria produced by ketogenic diets, cited above.

3. Ketonuria in the Early Stages of Fasting

Starvation ketonuria is generally thought of as a rather sudden phenomenon, appearing on the 2nd or 3rd day of fasting, presumably when the carbohydrate reserves of the body have become depleted. Benedict (8) reported 0.5 gm. of β -hydroxybutyric acid on the 2nd day of fasting. Folin and Denis (9), in their experiments on fasting in obesity, found 120 mg. of acetone and diacetic acid, but no β -hydroxybutyric acid, in the urine of one obese woman on the 1st day of fast, but their second subject, still more obese, excreted none of the acetone bodies until the 3rd day. Goldblatt (10) points out that the time at which acetonuria begins in fasting depends upon the diet previous to the commencement of the fast. In his experiments the time at which ketosis appeared, as indicated by the nitroprusside reaction, was 20 hours after the beginning of the fast, when the previous diet had been low in carbohydrates, and 36 hours after the last food, when an average mixed diet had preceded. Goldblatt speaks of the appearance of ketosis as an acute phenomenon, the concentration of acetone bodies rising from 3 or 4 mg. per cent (still negative to the reaction with nitroprusside) to 20 or 30 mg. per cent within 15 minutes.

Using the normal daily variations in ketone body excretion as a basis, we have studied the earliest stages of an increase in this excretion due to lack of food in a normal individual. Some of the results are shown in Charts 3 to 5.

The subject of these experiments was a woman, 38 years old, 5 feet, 7 inches in height, weighing 54 kilos, whose normal diet was not much in excess of the maintenance requirement and contained relatively little protein. The subject was in good health and active, showing no tendency towards glycosuria and giving normal acetone curves.

In Chart 3 a control period is shown with normal diet and normal ketone excretion. On the following day lunch was omitted.

was omitted, both the acetone-diacetic acid and the β -hydroxybutyric acid fractions showed an increase relative to the urinary volume (2 mg. per cent of acetone in each fraction) and the acetone and diacetic acid excreted per hour were also slightly above normal. During the following period, from 6 to 12 hours after breakfast,

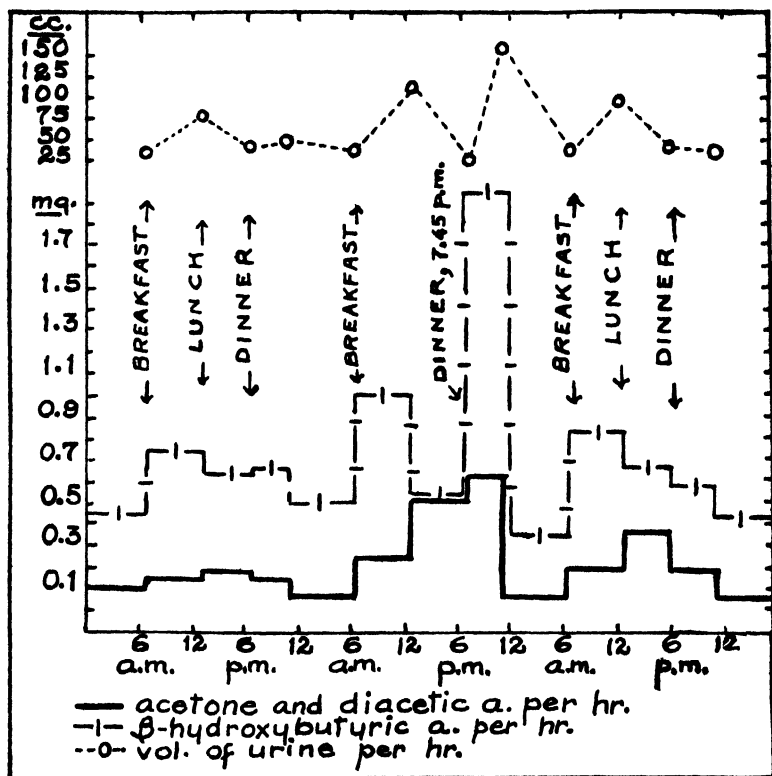


CHART 4

although dinner was eaten in the meantime, both fractions were increased per hour, the increase being paralleled by an increase in urinary volume. On the following day three rather light meals were eaten and the β -hydroxybutyric acid remained within normal limits but the acetone-diacetic acid fraction showed a tendency to rise again in the afternoon and evening.

These results were practically duplicated in another experiment which is not reported here. In still another experiment no significant increase in either fraction occurred on the day when lunch was omitted, but on the following day, a normal dinner and breakfast having been eaten in the meantime, both fractions increased dur-

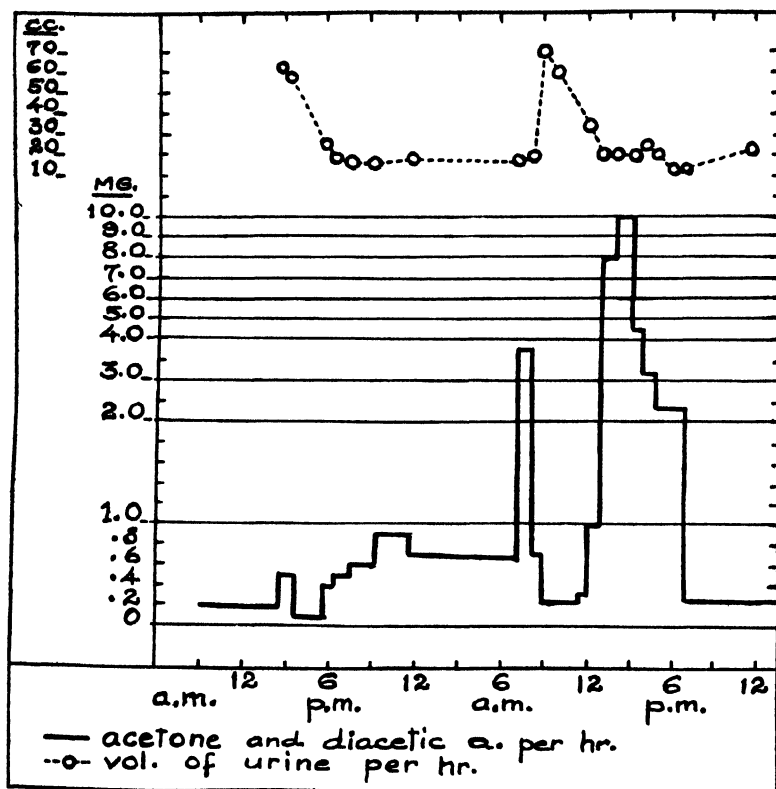


CHART 5. 1st day, 7 a.m.: normal breakfast; 12.10 p.m.: lettuce, mayonnaise, 2 cups of black coffee, 1 glass of water; 3.30 p.m.: 90 cc. of wine; 7.30 p.m.: 1 stearo cube in water, 2 gluten biscuits, 1 glass of water; 11.00 p.m.: 1 stearo cube in water, 1 glass of water. 2nd day, 7.30 a.m.: rice flakes with cream, 2 pieces of toast, butter, 2 cups of coffee, 2 lumps of sugar; 1.30 a.m.: 100 cc. of water; 3 p.m.: 15 cc. of whisky in 1 glass of water; 6.30 p.m.: normal dinner.

ing the period from 5 to 7 hours after breakfast (the acetone and diacetic acid to 0.9 mg. and the β -hydroxybutyric acid to 1.07 mg. per hour), returning to normal after lunch at 1.30 p.m.

In another experiment this subject ate almost nothing (practically no carbohydrates) for 30 hours, as follows: *1st day*: 7 a.m., normal breakfast; 1.15 p.m., 1 cup of pea soup, lettuce, mayonnaise, 1 cup of black coffee; 7 p.m., 2 cups of bouillon, lettuce, mayonnaise, 5 strips of bacon, 1 gluten biscuit; *2nd day*: 7 a.m., 1 orange, 1 gluten biscuit, 2 cups of black coffee; 1.30 p.m., 1 slice of bread, cole-slaw, 2 slices of canned pineapple, coffee with sugar; 7 p.m., normal dinner. On the 1st day the acetone and diacetic acid rose to 0.5 mg. per hour (2 mg. per cent) from 6 to 12 hours after breakfast and to 4 mg. per hour (15 mg. per cent) from 12 to 16 hours after breakfast. During the night the figures dropped to normal but on the following day rose to 2.5 mg. per hour (10 mg. per cent) between 10 a.m. and noon, and to 3.4 mg. per hour (20 mg. per cent) between noon and 1 p.m. During the 2 hours following the light carbohydrate lunch the acetone and diacetic acid fell to 1.75 mg. per hour (3 mg. per cent) and during the next 2 hours to 0.16 mg. per hour (1 mg. per cent). From 4 to 6 hours after the lunch they rose again to 2.8 mg. per hour (12 mg. per cent) and did not return to normal until 4 hours after the normal dinner.

Chart 5 illustrates an experiment on the same individual when practically no food was taken for periods of first 24 and then 12 hours. The experiment also shows the quick, temporary, anti-ketogenic effect from alcohol on this type of ketonuria. The food intake is recorded in the chart legend. The acetone and diacetic acid increased slightly from 7.5 to 8.5 hours after breakfast on the 1st day, and fell to normal during the 2 hours which followed the intake of 90 cc. of wine (about 8 per cent alcohol), independent of urinary volume. This was followed by a gradual increase in acetone and diacetic acid, which was only slightly diminished during the night. On the following day there was a still greater increase, which amounted to 10 mg. per hour (40 mg. per cent) at 3 p.m., when 15 cc. of whisky (about 40 per cent alcohol) were taken. During the following 3 hours there was a gradual decrease to 2.4 mg. of acetone per hour. Normal figures were not obtained, however, until after dinner.

A series of observations were also made on another subject, a

man 5 feet, 8 inches in height, weighing 91 kilos, whose normal diet was very liberal. He was active and in apparently good health. This subject habitually omitted lunch on alternate days, but the other meals on these days were large. The control periods on these days showed no abnormal excretion of ketone bodies. During two periods, however, when, after omitting lunch, this subject ate a large dinner, containing only green vegetables and fats, and again omitted lunch on the following day, there was a slight rise in both ketone body fractions during the evening and night of the 1st day, and an earlier rise on the following day. These figures are not reported in detail because the conditions of the experiments made no clear cut interpretation of the results possible. They seemed to show, however, that even in a well nourished individual a slight increase in ketone body excretion above the normal may occur very shortly after a decrease in food (especially carbohydrate) intake.

Certain points in connection with these experiments call for comment.

1. The first stages of abnormal ketonuria may be observed in some individuals earlier in fasting than was noted by Goldblatt, even as early as from 6 to 8 hours after food ingestion. An increase above the normal is noticeable before the ketone body excretion reaches the point at which a positive nitroprusside test is given. Goldblatt (10) evidently considered this point equivalent to not less than 3 or 4 mg. of acetone per 100 cc., which is considerably above the average normal figure. We are inclined to believe that although the acetonuria of fasting may increase in very rapid strides it is not a sudden phenomenon but may be traced as a development from the normal level.

2. It is difficult to correlate an increase in ketone body excretion so early in fasting with any extensive depletion of carbohydrate reserves. Other evidence may be adduced to show that abnormal ketonuria may occur before a maximum depletion of these reserves has taken place. The fasting subject of Benedict (8) excreted considerable amounts of β -hydroxybutyric acid on the 2nd day of fasting although carbohydrate was still being extensively utilized on the 3rd day. The carbohydrate catabolism decreased suddenly on the 4th day, which evidently marked the reduction of the carbohydrate reserves to their fasting minimum, but during the

first 3 days of the fast there was a slight decrease in carbohydrate catabolism although fat and protein catabolism did not decrease. It seems probable that even during the earliest hours of fasting there is a gradually increasing ketogenic-antiketogenic ratio in the foodstuffs metabolized, which results in an increased production of ketone bodies, at least in some part of the body.

3. The time at which an increase in ketone body excretion becomes noticeable in fasting is evidently correlated to some extent with the nutritional condition of the subject (Goldblatt (10)). The subject of these experiments was probably closer to the borderline of fasting than one whose normal diet was more in excess of a maintenance requirement. In this individual the intake of small amounts of food, low in carbohydrates, delayed the increase in ketone body excretion several hours. A reduction of food on one day led to an earlier increase on the following day. The other subject, whose normal diet was much greater, developed fasting ketonuria more slowly. Variations in basal metabolism and in energy expended during the experiments must also be important factors in determining the speed with which the increase in ketone body excretion becomes noticeable. The tendency toward a decrease in ketone body excretion during the night in these experiments seems to be in line with this.

4. It has been stated that the early appearance of fasting ketonuria is favored by a restriction of fluid intake (Goldblatt). This would seem to indicate a difference in mechanism between normal and fasting ketonuria. No attempt was made to study this point in our experiments and some water was taken during all of them. The acetone and diacetic acid tended to rise steadily during the day of fasting irrespective of urinary volume. Of the periods studied the one in which the rise appeared earliest was not the one in which the urinary volume per hour was lowest (Chart 3). The highest figures which were obtained, however, occurred when the urinary volume was unusually low (Chart 5), and an unexpectedly low acetone figure was sometimes found in connection with a high urinary volume.

5. In these early stages of fasting the β -hydroxybutyric acid of this individual tended to increase somewhat more slowly than the acetone and diacetic acid, and variation in its excretion seemed to be somewhat more closely correlated with variations in urinary volume.

6. The antiketogenic effect of alcohol upon fasting ketonuria, shown in these experiments, is in line with the finding that alcohol can replace fat in its sparing action on protein in normal subjects (Neumann (11), Rosemann (12), Atwater and Benedict (13)) and that it is selected by the body for oxidation in preference to carbohydrate and fat when it is fed with them (Cushny (14)). It is not in itself ketogenic. Diabetic ketosis is evidently little, if at all, affected by alcohol (Allen and Wishart (15), Joslin (16)). Evidently diabetic and fasting ketonuria are not comparable in this respect.

4. *Insulin and Diabetic Ketonuria*

In addition to the preceding discussion of normal ketonuria we would like to mention briefly certain points which have been brought out by a study of the urine of two diabetics during insulin treatment.

It has been well established that in practically all cases the excretion of ketone bodies by diabetics is reduced by insulin treatment, but the extent to which ketonuria becomes truly normal, and the time relationship between the lowering of sugar and of ketone bodies in the urine, after insulin, are points which might well be further investigated. The fact that has been of particular interest to clinicians is the reduction of ketone bodies in blood and urine to amounts below the level of severe acidosis. Most of the reports in the literature on this subject are based upon the results of qualitative tests. Examination of the reports in which quantitative methods have been used shows that the lowest figures reached after insulin treatment are still in most cases above the normal (Campbell (17), Fonseca (18), Chaikoff *et al.* (19), Killian (20), Davies *et al.* (21), Fitz, *cf.* Joslin (16) p. 59). There is also some indication that ketone bodies may decrease more slowly and reappear in the urine more rapidly than glucose, after insulin treatment, although this has not always been corroborated.

The two cases which we wish to discuss briefly at the present time were both hospitalized diabetic women who were receiving insulin three times a day before meals and whose urine we analyzed over extended periods.² In both cases the daily curves for the

² We are indebted for these samples to Mrs. Horning, of the laboratory of the Bethesda Hospital.

percentage concentration of urinary acetone and diacetic acid were remarkably similar from day to day, and were in contrast to the normal concentration curves, showing an increase during the night to a maximum in the morning, averaging 8.4 mg. per cent in one case and 11.5 mg. per cent in the other, and a gradual decrease during the day to their lowest point (0.1 mg. per cent in one case, and 2 mg. per cent, in the other) during the 6 hours which followed the last insulin treatment of the day. There was an apparent summation effect from the three insulin treatments. In one of the cases, in which the rate of acetone and diacetic acid excretion per hour could also be calculated, a similar tendency was found in the hourly excretion, although other variations occurred, which were evidently correlated with variations in urinary volume.

Of particular interest was the fact that in one case the glycosuria was completely controlled by the insulin treatments, the figures for urinary sugar never rising above the normal, but that nevertheless the acetone and diacetic acid figures approached the normal range only during a brief period late in the afternoon or evening, and at all other times were distinctly above the normal. In the other case the urinary sugar was not controlled by the insulin, showing only slight variations, between 0.7 and 1.2 per cent, which had no apparent relation to the insulin treatments or to the variations in urinary acetone and diacetic acid.

Such cases illustrate the variations in ketone body excretion which may occur under continued insulin treatment. We plan to continue this type of study.

5. Methods Used for the Determinations

Ketone bodies were determined as acetone in distillates from urine by a method which involves the reaction of acetone with salicylic aldehyde in strongly alkaline solution (22).

Acetone and diacetic acid were determined by a single distillation from one portion of urine which had been acidified with sulfuric acid. The volume of urine used and the volume of distillate collected were varied according to the amount of acetone present. It was found that the most accurate results are obtained when the original volume in the distilling flask does not exceed the volume

of distillate to be collected by more than 10 or 15 cc. In order to obtain a sufficient concentration of acetone in the distillate for the determination it was sometimes necessary, however, to distil a volume of only 10 cc. from an original volume of as much as 50 cc. of urine, no water being added to the contents of the distilling flask. In such cases slight traces of acetone remain undistilled, but the error involved is so small that it seemed justifiable to use this procedure as a means of obtaining normal urinary figures which are very nearly, if not quite, accurate, and which have a relative, if not an absolute, value. The limits of accuracy of the method may be considered approximately ± 0.03 mg. per 100 cc. of urine.

β -Hydroxybutyric acid was determined in another portion of urine after the removal of interfering substances by treatment with calcium hydroxide and copper sulfate as described in the regular method (22). In order to keep the volume as low as possible, equal volumes of urine and 40 per cent copper sulfate, without additional dilution, were made alkaline with solid calcium hydroxide. It was found that the mixing of these substances is made easier if an approximately correct amount of the hydroxide is mixed with the urine first and the copper solution then added with shaking. More calcium hydroxide may then be added if necessary, to give the mixture a bright blue color and an alkaline reaction to litmus. 30 cc. of urine, with an equal volume of 40 per cent copper sulfate, require about 5 gm. of calcium hydroxide, 100 cc., about 15 gm. The mixture was allowed to stand, with occasional shaking, for about 40 minutes and was then filtered with a little suction. The use of suction results in the loss of from 20 to 30 per cent of the acetone, so that it was always necessary to determine the acetone and diacetic acid in a separate portion of urine, which required less time than filtering the mixture without suction. A measured volume of the filtrate was then distilled with acid and bichromate as described in the regular method (22), the volume of the distillate being kept as low as possible (to between 40 and 60 cc.). If necessary because of the low concentration this distillate was then acidified and redistilled to a smaller volume (from 25 to 35 cc.).

The determination of acetone in the final distillates was made either as described in the regular method, or by the shorter method

described more recently, which involves the use of permanent color standards (23).³

Sugar determinations were made by Benedict's clinical quantitative test (24).

SUMMARY

Figures are presented for the normal excretion of ketone bodies and for the variation in this excretion during the day. The highest excretion occurred during the afternoon and evening. To a certain extent the excretion of ketone bodies per hour was correlated with urinary volume per hour.

The appearance of increased excretion of ketone bodies in the early stages of fasting is discussed and experiments are described which show that a slight increase above the normal may be noticed in an apparently normal individual as early as from 6 to 12 hours after the last meal.

The variations in urinary acetone and diacetic acid of two hospitalized diabetics receiving insulin are briefly described. The concentration of these substances showed characteristic daily curves with figures above the normal even when the urinary sugar was maintained within normal limits.

We wish to express our sincere appreciation to Dr. William Muhlberg for his cooperation and interest throughout this work.

³ The constancy of the color given in the acetone reaction by many different samples of Eimer and Amend's Acid Salicylous, Synthetic, obtained throughout a period of several years, made it seem possible to recommend the use of permanent color standards in this method. Since the completion of the work described in this paper, Eimer and Amend have discontinued the manufacture of this product and the imported product which they are now putting out under the same label gives slightly less color with acetone solutions than the former product. The difference is apparently negligible for practical purposes but it renders the advisability of the use of permanent color standards somewhat doubtful. This difficulty is not encountered when acetone solutions are used for standards as described in the method of Behre and Benedict (22). The new Eimer and Amend product has been found unsatisfactory for use in the qualitative test (4) probably due to the fact that it forms a less soluble mixture with sodium hydroxide than the former product. It can be used in this test with potassium hydroxide. The technical salicylic aldehyde of the Eastman Kodak Company has been found entirely satisfactory for use in the qualitative test, and is recommended for this.

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FREE AND BOUND WATER DETERMINATIONS BY THE HEAT OF FUSION OF ICE METHOD*

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Bound Water in Tissues

The dissolved materials and the colloids which are present in the water of the tissues have the effect of modifying some of the properties of the water in which they occur. As a consequence the water of the tissues does not behave in all respects like water in its pure state, and it is commonly spoken of as "bound" water.

Any variations in the concentration of substances in solution and any changes in the water-binding capacity of the colloids, which take place as a result of the processes of normal physiology or of pathology, may affect the degree of force with which the water is bound. Thus there is the possibility (Gortner, 1929) that the shifting which occurs, either backward or forward, in the water-binding may have some bearing upon biological processes.

The conception has been held by some investigators in this field that part of the water in the tissues is bound and the remainder free and that an equilibrium is maintained between these two conditions. According to other theories¹ all the water in the tissues is bound; and changing conditions affect only the force with which it is bound. Under either of these view-points, however, the method of Rubner for making bound water determinations can be applied to any series of comparative tests.

* The method of Max Rubner (1922).

¹ The writer is indebted to Dr. D. R. Briggs of this Institute for the use of his manuscript, as yet unpublished, on the theories of bound water in the tissues.

Principles of Rubner's Method

The fact is recognized that the colloids and dissolved materials hold water with an actual tenacity and that they resist any force which tends to pull the water away. In Rubner's method a definite and constant desiccating force is used, which tends to withdraw the water. That part which can be withdrawn under a standard desiccating force is called "free" and the remainder which is retained is termed "bound" under those conditions. This is an arbitrary distinction, but so long as the dehydrating force is constant this terminology serves in any series of comparative tests.

The method to be described in detail, although modified from the description given by Thoenes (1925) and by Robinson (1928) does not differ materially from the principles of the method of Rubner. A known weight of specimen, preferably between 0.4 and 0.8 gm. is placed in a prepared tin foil container of known weight, frozen at a constant temperature of -20° for several hours, and transferred to a calorimeter where a determination is made of the number of calories of heat required to melt the ice formed within the tissues. This determination is based upon the fact that to melt 1 gm. of ice without raising its temperature requires 80 calories of heat. By calculation the amount of free water per gm. of solid is determined. The final step in the method is to dry the material to constant weight as a measurement of total water content. The difference between the total and the free water values indicates the amount of bound water in the specimen.

In the freezing process an efficient desiccating force is provided since at temperatures below zero water tends to leave the colloids and dissolve materials to form ice crystals of pure water. The force exerted by this means is definite and constant at any given temperature below the freezing point of the tissue and increases with fall in temperature. On the other hand, the tendency of the water to crystallize is retarded by the dissolved and colloidal substances present. Therefore at any given temperature below freezing the amount of water which crystallizes out is a measure of the water-binding capacity of the tissue.

Accuracy of Method

With the use of the technique as described, results with homogeneous material may be obtained which are repeatable within a small range of error. Deviations from the mean were determined with 10 per cent gelatin in water. Small cubes were cut from the gel and determinations of total and bound water made. The average total water content was 8.344 gm. per gm. of dry gelatin, with a deviation of ± 0.001 gm.; and the average bound water was found to be 1.210 gm. with a deviation of ± 0.006 gm. In the case of biological tissues the range has been from 2 to 6 times greater. This is not necessarily because of inaccuracies in technique, since the assumption should probably not be made that such tissue is uniform throughout in its water content.

Inner Container

An inner container is essential in handling and weighing the material, and in preventing the following: (1) evaporation of water from the specimen; (2) heat of solution of any soluble substances while in the calorimeter; (3) floating of specimen on surface of water in calorimeter.

Tin foil is especially satisfactory for this purpose, and a fairly heavy foil such as 0.0015 inch thickness, which is readily obtainable, is recommended. Care should be taken to avoid foil with pin-holes.

The tin foil is given a *very light* coat of vaseline upon one surface, cut into pieces about 90 mm. square, and made up into small cylindrical cups. The cups may be made conveniently by pressing the middle of each square tightly around the base of a vial or metal rod about 12 mm. in diameter and then trimming it down to the required height of about 20 mm. The vaseline surface should be inside.

Outer Container

Each tin foil cup is placed in an outer glass container, partly for identification but principally to permit transfer of the specimen from the freezing cabinet to the calorimeter with the minimum of exposure to room temperature. A convenient container is the ordinary glass shell vial, approximately 18 mm. wide and 55 mm. high. Each vial is given a number which can be scratched on

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with a carborundum pencil, and each cork is plainly marked on top with the corresponding number.

The glass container, without the cork, and the tin foil cup are each weighed correct to mg. The cup is placed inside as soon as weighed and the vial corked.

Work Sheet

Because of the amount of data necessary for each determination, a separate work sheet has been found essential for each specimen. The form of work sheet shown is the kind used by the writer.

The weights of the glass and tin foil containers are recorded opposite (1) and (2) respectively. All weights recorded should be accurate to mg

Work Sheet

FREE AND BOUND WATER DETERMINATIONS

Container No	Material	Date	
S	= Specific heat of material		
S_1	= " " " tin foil	0 05	$FN (T_2 - T_3)$
	Weight of container + W_1 + dry W	mg (5)	WSR
	Weight of glass container	" (1)	$W_1 S_1 R$
	" " W_1 + dry W	(6)	
	" " W_1 + total W	(3)	
W_1	= " " tin foil covering	(2)	Drying weights (5)
W	= " " material	(4)	mg
	Dry weight of material	(7)	"
	Total water per cent	(8)	"
X	= Free "	" (9)	
	Bound " per cent	" (10)	
	Current generated in thermopile		microvolts per degree
T_1	= Initial temperature of material	-20 0	degrees
T_2	= " " " water in calorimeter		microvolts
T_3	= Final temperature of water in calorimeter		"
R	= Range of temperature between T_1 and T_3	$= 20 0 + () = ()$	
	$T_2 - T_3 =$	microvolts	degrees
F	= Correction for thermal capacity of calorimeter		
N	= Volume of water in calorimeter	10	cc
X	= $\frac{FN (T_2 - T_3) - (WSR + W_1 S_1 R)}{80 - \frac{T_1}{2}}$		Water per gm dry wt { Total water mg Free " Bound " "

Remarks

Freezing Apparatus

The determination of bound water by the present method requires thorough freezing of the material at a constant temperature. The value for bound water obtained varies with temperature and thus it is important to maintain the freezing temperature constant, preferably within 0.25° . A temperature of -20° was suggested by both Rubner and Thoenes and is the one used by the writer. A somewhat higher one may be used provided it is maintained constant throughout any series of comparative tests. A suitable freezing mixture held in a well insulated vessel may possibly serve the purpose for short experiments if especial care be taken, but such a method is not recommended.

Best results are obtained with a low temperature cabinet attached to a mechanical refrigerator. The cabinet is made to open on top to prevent temperature changes when the door is opened. The dimensions of the freezing compartment will depend upon requirements: probably 12 inches square and 24 inches deep will be suitable for most purposes. This compartment is best made entirely of sheet copper. A brine or alcohol bath surrounds this compartment on all four sides, and the whole is insulated with about 6 inches of cork. If brine is used, approximately 1 pound of hydrated lime to about 30 gallons of brine is added to prevent corrosion of the container. The expansion coils from the compressor are installed in the bath. The cabinet should be connected to the compressor by the company that supplies the refrigerating unit.

Temperature Control

A constancy of temperature within 0.25° is beyond the limits of most mechanical refrigerators. It is necessary, therefore, to install a reliable thermoregulator in the cabinet. This should be placed in the freezing compartment and not in the brine bath.

As the compressor motor will probably require at least 5 amperes in starting, most thermoregulators will not be able directly to pass a current of this magnitude, and a suitable relay must be connected in the circuit of the compressor motor to make and break the current to the motor. In that way only the weak current necessary to activate the motor relay will pass through the thermoregulator. The control supplied on the compressor unit

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should either be discarded or adjusted so that it always allows the motor circuit to remain closed.

When the cabinet is in proper working condition the specimens for determination are placed in their tin foil cups, weighed, and recorded at (3) on the work sheet, and then frozen. The net weight of the material is obtained by subtracting (2) from (3). It has been found advisable to limit the net weight of the specimens to between 0.4 to 0.8 gm., larger amounts causing a small error through delayed equilibrium with the water in the calorimeter.

The length of time a specimen is allowed to remain frozen appears, from tests made at this laboratory, to have no effect upon the amount of water bound. Therefore material may be kept in the frozen condition, in which postmortem changes will probably not occur, and determinations made as convenient.

The Calorimeter

This is a silvered Dewar flask. Dimensions suitable for tin foil cups as specified are about 65 mm. high and 12 mm. wide, inside measurements. When a determination is being made, the flask should not be touched with the warm hands but should be held in a condenser clamp with rubber-covered jaws. The calorimeter is closed with a cork about 15 mm. thick.

Calorimeter Bath

Determinations should be made in a room free from sudden or great changes in temperature. As an added precaution it is well to immerse the calorimeter close to the top in a water bath standing at room temperature.

Amount of Water to Use in Calorimeter

For specimens of tissue weighing from 0.4 to 0.8 gm., 10 cc. of water are sufficient to use in the calorimeter. The initial temperature of the water, T_2 , should be approximately as much above that of the bath and room as the final temperature, T_3 , falls below it. The water is measured out accurately in a volumetric pipette. Before each determination the inside of the calorimeter is dried with absorbent gauze.

Stirring of Water in Calorimeter

One of the greatest sources of error can exist at this stage unless stirring is thoroughly done. This is true especially after the transfer of the material to the calorimeter. Horizontal whirling is not sufficient and thorough vertical mixing of the water is essential. Glass being a poor conductor of heat is the best material to use for the stirring rod. A piece of tubing about 4 mm. in diameter is heated and flattened at one end for a length of about 30 mm. and twisted into a spiral. Care is taken to keep the rod in alignment throughout so that when rotated it will occupy its smallest space inside the calorimeter. The rod is attached to a small motor stirrer having an adjustable speed.

The specimen, when transferred to the calorimeter, sinks and cools the water at the bottom. The cold water being denser resists mixing with the warmer layers on top, and care must be taken to see that the stirrer overturns and mixes the water completely. A speed of 2000 or more revolutions per minute may be necessary with a stirring rod as specified. The speed of stirring should be sufficient to overturn the water without whirling the specimen. If the specimen is whirled it will strike against the thermocouple and cause difficulties in temperature determination.

In finding the speed necessary, a good method is to cut a number of pieces of filter paper about 1 mm. square and after moistening allow them to sink to the bottom of the water in the calorimeter before the specimen is transferred. Then after the transfer has been made the paper will indicate whether the cooling water is being stirred sufficiently to overturn it.

The stirring of the water adds a minute amount of heat, but this is taken into account automatically when the thermal capacity of the calorimeter is determined.

Method of Determining Temperature of Water in Calorimeter

The temperatures, T_2 and T_3 , should be determined to 0.01° , which requires a thermometer of more than ordinary precision. The thermocouple has so many advantages over the mercury thermometer for this purpose that it is unquestionably the most satisfactory instrument to use; and its use is strongly recommended. The single thermocouple, however, especially when used

with a galvanometer of low sensitivity, cannot be relied upon to indicate 0.01° accurately. The compound thermocouple called a thermopile, made up of several simple thermocouples in series, greatly increases the magnitude of deflection of the galvanometer and makes possible reading small parts of a degree with satisfactory accuracy. A thermopile described by Robinson (1931) for entomological work will be found suitable for this purpose.

In the cork which closes the top of the calorimeter a slot is cut wide enough to accommodate the stem of the stirring rod without touching. Also a hole is bored to take the thermopile bundle, which is pushed through far enough to reach well into the water without coming in contact with the specimen. The bundle is then set permanently into place in the cork.

Capping the Specimens

The tin foil cup should be high enough to press down tightly on top and completely enclose the specimen. With solids the capping is done at once but in the case of liquids the cup is closed temporarily by pressing the sides together at the top, final capping being done after freezing and at least 1 hour before making the determination.

Transferring Specimens

The required volume of water is placed in the calorimeter carefully so that none is splashed on the sides. Its temperature is then determined, with the stirrer going, and recorded as T_2 . The motor-stirrer is next stopped and the specimen removed from the freezing cabinet and instantly transferred to the calorimeter through the glass container. This should be done as quickly as possible and without touching the specimen with the fingers. The stirring is then resumed.

In transferring, the glass vial is held by the top to prevent any possible warming of the material by the fingers. The thermopile bundle attached to the cork is removed from the water just long enough to permit transfer of the specimen. The bundle will serve also to guide the specimen into the calorimeter.

The speed and skill with which the transfer is made will largely affect the uniformity of results obtained. The point at which the

temperature of the water in the calorimeter, with the stirrer in operation, ceases to fall and remains stationary for a time is then recorded as T_3 .

Drying Specimens for Total Water Content

The specimen is removed from the calorimeter and returned to its numbered glass container, in which the tin foil cup is pierced to hasten drying. When a sufficient number have been brought to this stage they are uncorked and dried in an oven.

Drying can be done in the ordinary type of laboratory oven, at 100° , or in a vacuum oven at a much lower temperature, say, about 60 to 65° . Drying is continued until a constant weight is obtained which is recorded at (5) on the work sheet. This includes the weight of the glass container, the tin foil covering, and the dry weight of the material. From that can be determined the dry weight (7) and the total water (8).

Calculation of Free and Bound Water

Free water, X , is calculated according to the following formula.

$$X = \frac{FN(T_2 - T_3) - (WSR + W_1S_1R)}{80 - \frac{T_1}{2}}$$

where F = correction for thermal capacity of calorimeter

N = volume of water in cc. used in calorimeter

T_2 = initial temperature of water in calorimeter

T_3 = final temperature of water in calorimeter

W = total weight of material

S = specific heat of material

R = range in temperature between T_1 and T_3

W_1 = weight of tin foil covering

S_1 = specific heat of tin foil which is 0.05

T_1 = initial temperature of material in freezing cabinet. This being below zero appears to have a minus value but the formula is constructed so that the sign may be disregarded.

To obtain the value for F , several 0.4 to 0.8 gm. samples of distilled water, accurate to mg., are prepared, frozen, and transferred to the calorimeter exactly as in bound water determinations.

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Calculation of the thermal capacity of the calorimeter is made according to the following formula.

$$F = \frac{W \left(80 - \frac{T_1}{2} \right) + WSR + W_1 S_1 R}{N (T_2 - T_3)}$$

The value obtained for F should theoretically be a constant, but at first will probably be found to vary somewhat. An average value such as 1.135 may be obtained, depending upon the size of the calorimeter and the amount of water used in it. A deviation of ± 0.003 from the average is sufficiently accurate. Thermal capacity will have to be determined for each calorimeter used, and also when any change is made in stirring rod or thermopile bundle.

The next determination to be made is that of the specific heat of the material. This may readily be done by the method of mixtures, as follows: Specimens are prepared with inner and outer containers as for bound water determinations but are placed in an ice box standing above freezing and well below room temperature. An accurate thermometer is inserted into a corked and empty vial and placed in with the specimens, and temperature is noted for each specimen when the transfer is made. After 3 or 4 hours each specimen is transferred quickly to the calorimeter and allowed to come into equilibrium with the water as described for bound water determinations. Care must be taken to grasp the vial at the top when making the transfer, as previously advised. Owing to the short range between T_2 and T_3 in specific heat determinations, the precaution is especially necessary to have these two temperatures as nearly as possible equidistant from the bath temperature. T_3 should never be above that of the bath, otherwise it would be difficult to obtain. The material is finally dried in the oven for total water content.

Specific heat, S , can be calculated by the following formula.

$$S = \frac{FN (T_2 - T_3) - W_1 S_1 (T_3 - T_1)}{T_3 - T_1} \times \frac{1}{W}$$

Determinations of S should be made upon several samples and the average value used. With a material having a uniform water content results may be obtained which deviate from the average by not more than ± 0.02 .

Specific heat varies not only with different materials but also with their total water content. If bound water determinations are to be made with a tissue whose total water varies considerably, it is possible to avoid making a large number of determinations of S and to arrive at all its values through a simple formula, the specific heat of the dry weight or solid fraction being used.

$$S = h_w f_w + h_s f_s$$

where h_w = specific heat of water which is always 1.0

f_w = fraction of total weight which is water

h_s = specific heat of dry weight or solid

f_s = fraction of total weight which is solid

This formula has been made available to the writer through the courtesy of Dr. D. R. Briggs (1931) of this Institute.

Determinations of h_s made directly with dried material are not recommended as it is a less accurate procedure than when the total weight is used. A preferable method is to make the original determinations for S as described, typical samples of fresh tissue being used; then to calculate h_s by means of a rearrangement of the previous formula, as follows:

$$h_s = \frac{S - h_w f_w}{f_s}$$

This indirect method of arriving at the specific heat of specimens gives a close approximation of their true value and saves much time when water content varies.

The final step in the Rubner method is to calculate the free water, X . This may be recorded at (9) on the work sheet. Subtracted from the value for total water, it indicates the amount of water (10) in the bound condition at the freezing temperature used.

The use of a slide rule will be found to save a great deal of time when calculations are being made.

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RICKETS IN RATS

XII. THE ACID-BASE EQUILIBRIUM OF THE BLOOD IN RICKETS AND TETANY

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The significance of acids and alkalies in rickets and tetany is not clear because evidence of different types has often been confused. The problem would be clarified if it were divided into at least three fields of inquiry, the etiology, therapy, and pathogenesis. Does acid or alkali feeding cause rickets or tetany? Does acid or alkali therapy result in a cure of rickets or tetany? Is rickets or tetany associated with a constant alteration in the acid-base equilibrium of the blood? When these questions have been answered clearly, the part of each in the physiological pathology may be evaluated.

In the early studies, it was concluded that acid caused rickets. These were made before rickets and osteoporosis were separated. The low calcium content of the body caused by the depletion of body bases was interpreted as rickets. Histological studies have shown that this view-point is incorrect. Modern studies have demonstrated that rickets in rats is produced by a lack of vitamin D or ultra-violet light associated with a defect in the calcium and phosphorus content of the diet. The importance of the acid-base factors has been investigated. McClendon (1) maintains that alkaline diets cause a more intense rickets. Zucker (2) states that a ricketogenic diet is converted to a non-rickets-producing diet by the addition of acids. However, McCollum (3) and Shelling (4) have produced rickets of equal intensity upon both alkaline and acid diets.

In tetany the situation is still more confusing. Tetany can be

produced in a number of ways; infantile idiopathic tetany by the inadequate or interrupted therapy of rickets (5), by fasting ricketic animals, by hyperventilation, by removal of the parathyroids, and by administration of phosphate, of oxalate, of citrate, and of alkali in the presence of damaged kidneys. In only a few of these cases can the importance of the acid-base equilibrium be traced. Binger (6) demonstrated that, in dogs, alkaline phosphates given intravenously diminish the serum calcium and cause tetany but that phosphates more acid than pH 6.0 cause a lowering of serum calcium but no tetany. Salvesen, Hastings, and McIntosh (7), however, demonstrated that, in dogs, phosphates given by mouth cause tetany whether given as the acid, alkaline, or neutral phosphate. No one has ever demonstrated that in normal animals the feeding of alkalies other than phosphate results in tetany. In the presence of damaged kidneys and in the treatment of diarrheal acidosis Howland and Marriott (8) showed that intravenous alkali injection resulted in tetany. The evidence is therefore much clearer that tetany of *certain types* is produced by alkali than that rickets is the result of acid administration.

If rickets is due to acidosis, alkali therapy should result in the cure. Hess (9) has pointed out that such is not the case. Fasting produces an acidosis and fasting of ricketic animals has been shown (10, 11) to result in healing.

Tetany is undoubtedly relieved by acid feeding. This has been repeatedly demonstrated in infants by Scheer (12), Freudenberg and György (13), and Gamble *et al.* (14). Parathyroidectomized animals are improved by acid therapy (15). The evidence with regard to tetany is much clearer than for rickets; namely, that administration of acid leads to amelioration.

When rickets is present Hodgson (16) and Freudenberg and György (17) have maintained that acidosis is also present. The former reached this conclusion from a study of the titratable acid and NH_3 content of the urine, and the ability of the infants to take large doses of NaHCO_3 without rendering the urine alkaline. The latter have made similar studies and also measured the CO_2 content of the blood.

In gastric tetany, in hyperventilation tetany, and in the tetany produced by large doses of alkali in individuals with damaged kidneys, alkalosis is undoubtedly present. Wilson and coworkers

(18) thought alkalosis was present in parathyroid tetany. Van Slyke (19) went so far as to indicate by charts that tetany was present when the blood became as alkaline as pH 7.8. In no other form of tetany has alkalosis been proved. Certainly in infantile tetany the acid-base balance of the blood is within normal limits (Drucker, 1927 (20)). No one has demonstrated an alkalosis in the tetany produced by fasting ricketic animals.

It is clear, then, that in rickets, either from the point of view of etiology, therapeutics, or pathogenesis, the evidence of acidosis is not convincing. In tetany the situation is much more complex. The evidence is convincing that certain types of tetany are associated with an alkalosis. It is equally clear that other types of tetany are not associated with alkalosis. This manifold nature of tetany must be taken into account in any general explanation. However, until all the factors have been evaluated, such theoretical discussion must remain mere speculation.

Plan of Procedure

This study was designed to evaluate the acid-base equilibrium of the blood serum of rats with experimental rickets and tetany, and hence to determine the significance in these conditions of the acid-base factor in pathogenesis. For the production of rickets the well known high calcium-low phosphorus Diet 2965 of Steenbock and Black (21) was used. This contains CaCO_3 and its alkali value equals 630 cc. (0.01 N) per 100 gm. of diet. To compensate for this alkali normal controls were studied both on Sherman's Diet B (22), which is nearly neutral, and the same diet plus enough alkali to equal that of the rickets-producing diet. As a further control 15 mg. of cod liver oil were given daily by pipette to rats consuming the rickets-producing diet. Such animals are completely protected against the development of rickets.

From the various types of tetany we elected to study only those two associated with rickets; that produced by fasting and that produced by the increase of the phosphorus content of the rickets-producing diet to that amount which is considered normal for the calcium present. The phosphate added was Na_2PO_4 , NaH_2PO_4 , or H_3PO_4 sufficient to bring the ratio of Ca:P either to 2 or 1. Both of these forms of tetany, we have considered, are produced by phosphate; the source of the latter is exogenous and the former endogenous (11).

Methods

The breeding of the animals and their care was the same as that previously described (23). At least four animals were used in each group. The blood was pooled and analyzed in duplicate. Each experiment was repeated at least three times with results that were in close agreement. A few of the first experiments were rejected for obvious errors in technique and are omitted from the averages.

Rickets was diagnosed by x-ray pictures and the calcium and phosphorus content of the blood serum. Tetany was evidenced by the calcium and phosphorus content of the blood serum and by the galvanic electrical reactions of Shohl and Bing (24). The acid-base equilibrium of the serum was determined by the measurement of the pH and CO_2 . As confirmatory evidence the serum was analyzed for the chlorides, total base, and protein.

The blood was obtained without contact with air from the vena cava and received under oil. The vena cava was exposed either by abdominal incision under novocaine or after the rats were stunned lightly.

Calcium in the serum was determined by titration of calcium oxalate with permanganate, according to the method of Clark and Collip (25), phosphorus in the serum by the stannous chloride reduction of the molybdate, the method of Kuttner and Cohen (26) being used, pH and CO_2 , both on a single sample of 0.2 cc. of serum, by the method of Shohl (27). Chlorides were determined according to the modification of Van Slyke's method described by Wilson and Ball (28), total base by the method of Stadie and Ross (29), and serum protein by the macro-Kjeldahl method upon 1 cc. of serum.

Results

The data are given in Tables I and II. Each value represents the average of three or more concordant experiments. In rickets, the values for the acid-base equilibrium represent the upper normal limit and border on alkalosis. In tetany, whether produced by phosphates, whether acid, neutral, or alkaline, or by fasting, the pH, CO_2 , and total base of the blood serum are significantly diminished. Also, in the animals which received cod liver oil and hence did not have rickets, and in the animals which received the

most acid phosphate but had no tetany, acidosis was present. The values for the protein are given in Table III. They indicate that in younger rats lower protein values are encountered. The lowest values occur in the groups with rickets and those protected

TABLE I

Acid-Base Equilibrium of Blood Serum of Rats with Rickets and Tetany

Diet*	pH	CO ₂	[Cl]	[Cl] + [CO ₂]	Total base	Remarks
		vol. per cent	m.-eq.	m.-eq.	m.-eq.	
Sherman Diet B (10 cc. 0.1 N alkali).....	7.34	63.7	104	131	148	Normal
Sherman Diet B + NaHCO ₃ (510 cc. 0.1 N alkali).....	7.33	62.5	111	137	149	"
Steenbock and Black Diet 2965 + cod liver oil (530 cc. 0.1 N alkali).....	7.27	56.6	113	137	146	Protected diagnosis by x-ray
Steenbock and Black Diet 2965 (530 cc. 0.1 N alkali).....	7.40	64.3	106	133	148	Rickets diagnosis by x-ray
Same; Ca:P = 1 for 48 hrs. (860 cc. 0.1 N alkali)†.....	7.26	50.4	109	130	134	Tetany, low COC, convulsions

* The acid-base value is calculated per 100 gm. of diet.

† After 21 days on the rickets-producing diet, rats were given additions of phosphate as Na₂PO₄, H₃PO₄, and H₂PO₄ and HCl to give the indicated acidity and ratio of calcium to phosphorus.

from rickets, the highest in the groups with tetany. The variations are so slight as to be most likely within the probable errors and indicate that no significant dilution or dehydration has occurred. The base bound to protein was diminished in the serum of the animals with tetany.

TABLE II
Acid-Base Equilibrium of Blood Serum of Rats with Tetany

Diet*	pH	CO ₂	Serum Ca	Serum P	Remarks
		vols. per cent	mg. per cent	mg. per cent	
Fasted					
24 hrs.	7.30	58.7			Tetany, low COC, tremors and convulsions
48 "	7.23	56.0	6.7	9.4	
Ca:P = 2 (600 cc. 0.1 N alkali)					Tetany, low COC, tremors
24 hrs.	7.26	54.0			
48 "	7.23	56.5	5.7	11.3	" "
Ca:P = 2 (50 cc. 0.1 N alkali)					
24 hrs.	7.23	51.0			No tetany, normal COC, no tremors
48 "	7.24	62.0	6.8	9.3	
Ca:P = 2 (300 cc. 0.1 N acid)					
24 hrs.	7.24	51.6			
48 "	7.25	54.4	9.6	9.2	Tetany, low COC, convulsions
Ca:P = 1 (860 cc. 0.1 N alkali)					
48 hrs.	7.25	47.2	5.0	12.0	" "
Ca:P = 1 (50 cc. 0.1 N acid)					
48 hrs.	7.26	49.6	5.0	12.0	

* The acid-base value is calculated per 100 gm. of diet. After 21 days on the rickets-producing diet, rats were given additions of phosphate as Na₂PO₄, H₂PO₄, and H₃PO₄ and HCl to give the indicated acidity and ratio of calcium to phosphorus, or fasted.

TABLE III
Serum Protein in Rats

Normal		Rickets		Protected		Tetany	
Age	Protein	Age	Protein	Age	Protein	Age	Protein
	per cent	days	per cent	days	per cent	days	per cent
Adult	5.95	49	6.50	49	5.85	51	6.30
"	6.10	69	6.30	51	5.55	51	6.10
"	6.90	66	5.80	51	5.55		
31 days	5.70	66	5.70	51	5.45		
31 "	5.85	51	5.70				
51 "	5.74	49	5.40				
		52	5.95				

DISCUSSION

The acidosis found is accompanied by a diminished base. Hence the presence of lactic or other organic acids is not evidenced. The base bound to protein is significantly diminished. The findings in this study are so contrary to the common opinion that the burden of proof should correctly rest upon us. It will be necessary at the outset to state clearly that no attempt has been made to postulate whether similar conditions are found in rickets and tetany in other species or even in the rat for other diets or other forms of tetany than those specifically studied.

The diets customarily used to produce rickets are quite alkaline. The feeding of corresponding amounts of alkali has never been shown to result in an alkalosis. Infants on a milk diet also have an alkaline diet and may develop rickets.

The main question under investigation is not whether acidosis or alkalosis is an accompaniment of rickets or tetany, but whether it is an essential part. If rickets can be produced with either acid or alkaline diets and if tetany may result either with acidosis or alkalosis, then either is not a necessary feature. The most obvious hypothesis at present available is that tetany and rickets both result from an alteration of the activity or ionization of calcium and phosphate and that this may take place without alteration of either the total calcium or phosphorus or with a change in either direction of the acid-base equilibrium. Some of the factors which govern the ionization of calcium and phosphate and their precipitation as calcium phosphate and carbonate have been determined. However, it is our opinion that there are unknown factors operating in the blood serum, which are as yet impossible to evaluate. Undoubtedly increased phosphate and carbonate and alkali all tend to depress calcium ionization and therefore an increase in any of these three should operate adversely in both rickets and tetany, provided one assumes that diminished calcium ionization produces tetany and diminished ionization of calcium and phosphate produces rickets irrespective of the local factor. Viewed from this angle practically all of the forms of clinical tetany can be brought into line. For example, oxalate and phosphate depress the calcium ionization and should produce tetany. Gastric tetany causes an increase of the bicarbonate and alkalosis and should produce tetany. Hyperventilation produces alkalosis

which should produce tetany. In the case of our experiments and perhaps in many others the factors do not all operate in one direction. In our experiments with tetany the dominant factor seems to be the state of the calcium and phosphate of the blood; the acidosis is a secondary factor.

In our previous experiments (30) it was shown that alkaline phosphate given to ricketic rats produced less severe tetany than did neutral phosphate, whereas the most acid phosphates prevented the appearance of tetany. Although the acid-base values of the food eaten may not be reflected in their effect upon the organism, because of variation in absorption, acid feeding should cause an acidosis. Fasting certainly does not produce an alkalosis. This is strong evidence that alkalosis is not an essential feature of tetany. Whatever may be the interpretation on a theoretical basis, the facts seem to warrant the conclusion that rickets is not necessarily associated with an acidosis, and that tetany is not necessarily associated with alkalosis.

SUMMARY

In rickets the acid-base equilibrium of the blood may border on alkalosis. Tetany produced by fasting and phosphate feeding in rats previously made ricketic on a high calcium-low phosphorus diet results not in an alkalosis but in an acidosis.

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THE DISTRIBUTION OF CHLORIDE AND BICARBONATE BETWEEN PLASMA AND CELLS IN THE BLOOD OF VARIOUS PATHOLOGICAL CONDITIONS

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Van Slyke, Wu, and McLean (1) have considered the factors responsible for the unequal distribution of the diffusible ions between serum and cells by studying the blood at equilibrium after equilibration with known tensions of carbon dioxide and oxygen. Under these conditions it was found that the determined distributions could be approximately predicted by equations which were developed from the assumptions that the osmotic pressure is equal in cells and serum and that the distribution of the diffusible ions is influenced by non-diffusible ions according to the Gibbs-Donnan law. Previous to this report the distribution of diffusible ions between serum and cells was studied by Warburg (2) and it was concluded that the chloride and bicarbonate distribute themselves between the cells and serum in accord with the Donnan theory. In a later more critical study of the problem, Van Slyke, Hastings, Murray, and Sendroy (3) verified the earlier conclusions of Van Slyke, Wu, and McLean, and more recently Hastings, Sendroy, McIntosh, and Van Slyke (4) have obtained data on the distribution of chloride and bicarbonate in the blood of normal and pathological human subjects. The latter workers found that the r_{Cl} and r_{HCO} ratios did not deviate any more in pathological bloods at the same pH, than in normal bloods. This was taken as attributable to the fact that the two factors which are theoretically most important in determining the values of the ratios at a given pH, are the total base concentration in the serum and the hemoglobin concentration in

the cells and that neither of these showed great fluctuations in the conditions studied.

During the past 3 years, data have been accumulated on forty-eight hospital cases (Table I) which included the plasma concentrations of chloride, bicarbonate, pH, and water; and whole blood concentrations of chloride, bicarbonate, oxygen capacity, oxygen content, water, and cell volume. From these results the cell concentrations could be calculated and compared with the plasma concentrations. These data were collected to see whether abnormal distributions occurred in different pathological conditions with markedly differing cell volumes and electrolyte concentrations and also whether any change occurred in the course of a disease.

Methods

The blood in all instances was obtained before breakfast. Amounts up to 40 cc. were drawn under oil into a special centrifuge tube containing heparin as the anticoagulant. Enough whole blood was separated under oil for oxygen and CO₂ content, chloride, cell volume, water, and oxygen capacity determinations. The remainder of the whole blood was quickly centrifuged and the plasma separated and stored under oil. Plasma analyses of pH, CO₂, chloride, total protein, and water were made as quickly as possible.

The pH was determined colorimetrically by the method of Myers and Muntwyler (5). The Van Slyke and Neill (6) procedure was employed for the carbon dioxide and oxygen determinations. The Van Slyke (7) and the Wilson and Ball (8) procedures were employed for the chloride determinations. The water contents were obtained by drying to constant weight 1 cc. quantities of material, at 105°. The cell volume determinations were made without dilution in quadruplicate, Van Allen (9) hematocrit tubes being employed.

Calculations

The bicarbonate concentration of the plasma and whole blood was obtained by employing the following formula.

$$\text{mm BHCO}_3 = (\text{CO}_2) - \frac{(\text{CO}_2)}{10^{\text{pH} - \text{pK}'} + 1}$$

where a pK' of 6.10 and 6.13 was employed for plasma and whole blood, respectively. The (CO_2) represents total CO_2 in mm concentration.

The cell concentrations were obtained from the whole blood and plasma concentrations by solving the following statement.

$$\text{Whole blood} = \frac{p(100 - \text{cell volume})}{100} + \frac{c(\text{cell volume})}{100}$$

In order that the distribution ratios obtained with the different bloods could be compared, the values were corrected to the ratio corresponding to completely oxygenated blood by employing the following equation as was done by Hastings *et al.* (4).

$$c = 0.1 (pH_p - 6.6) \frac{\text{reduced Hb}}{\text{total Hb}}$$

where c is the amount by which the determined ratios must be decreased to give the ratios of completely oxygenated blood.

Results

The results of the analysis of 84 bloods on forty-eight hospital cases are presented in Table I. The cases include the following.

Chronic nephritis.....	7	Severe hypertension.....	16
Bichloride poisoning.....	6	Alcoholic jaundice.....	2
Nephrosis.....	2	Peritonitis.....	1
Cardiovascular renal dis-		Obesity.....	1
ease.....	2	Diabetes.....	1
Arteriosclerosis.....	1	Maternity.....	9

Considerable acidosis was present in five of the cases of chronic nephritis and in two of the bichloride poisoning cases. Comment should be made on the cases termed severe hypertension. In practically all of these subjects the systolic blood pressure was above 200 and in those observed over considerable periods of time the blood pressure remained persistently high. There was only slight blood nitrogen retention associated with the hypertension. The cases labeled maternity showed an elevation of blood pressure accompanied with a varying degree of proteinuria and only a slight blood nitrogen retention. All of the maternity samples were taken ante partum.

TABLE I
Chloride and Bicarbonate Concentrations in Plasma and Cells

Case No.	Day of observation	pH	Plasma			Cells		Whole blood H ₂ O	Cell volume	O ₂ capacity per liter of blood	O ₂ content per liter of blood	(Cl) _c (Cl) _p	(HCO ₃) _c (HCO ₃) _p
			Cl	HCO ₃	H ₂ O	Cl	HCO ₃					Corrected to 100 per cent oxygenation	Corrected to 100 per cent oxygenation
			mM per 1000 gm. H ₂ O	mM per 1000 gm. H ₂ O	gm. per l.	mM per 1000 gm. H ₂ O	mM per 1000 gm. H ₂ O	gm. per l.	per cent	mM	mM		
1	17	7.45	114.2	25.79	933	79.8	18.13	829	48.9	8.65	6.55	0.678	0.682
	27	7.46	113.0	27.95	941	78.3	27.71	820	46.4	8.44	5.80	0.666	0.750
	40	7.39	115.1	25.64	950	83.1	20.51	811	45.5	8.21	5.35	0.695	0.773
2	17	7.36	94.8	21.86	935	61.2	17.06	873	23.0	3.54	0.78	0.586	0.721
3	17	7.28	104.8	12.31	929	96.4	12.42	886	29.5	2.42	0.32	0.860	0.951
	24	7.18	105.4	11.00	934	88.4	10.29	892	22.8	3.65	1.88	0.811	0.907
	35	7.09	97.6	13.42	925	77.9	12.67	880	23.4	3.33	0.72	0.761	0.908
4	17	7.41	106.9	17.24	931	76.4	16.83	891	17.2	4.73	2.21	0.670	0.931
	8	7.23	104.6	17.68	911	76.4	17.73	861	20.0	3.31	1.40	0.690	0.960
	36	7.29	97.7	12.61	911	83.2	12.91	874	16.9	3.23	1.66	0.817	0.986
5	17	7.08	115.7	11.57	936	80.3	8.67	899	20.0	3.08	1.61	0.671	0.726
	8	7.28	106.2	14.31	951	85.8	13.25	891	18.9	2.82	0.92	0.768	0.885
	16	7.16	104.7	14.48	926	71.4	12.40	890	17.4	2.79	0.88	0.643	0.820
6	17	7.16	95.6	8.93	931	80.0	8.41	892	15.7	2.84	0.80	0.775	0.902
7	17	7.10	104.8	4.86	939	88.8	5.03	850	26.8	5.20	2.74	0.823	1.026
8	17	7.42	84.3	18.84	920	65.9	18.49	838	29.8	6.40	2.90	0.737	0.937
	8	7.41	82.8	20.94	911	60.4	17.46	826	42.2	8.44	4.32	0.689	0.794
9	17	7.50	106.3	31.75	924	71.3	22.90	817	50.0	8.80	5.26	0.635	0.685
10	17	7.07	88.2	6.89	929	91.9	8.82	794	37.2	6.08	4.65	1.029	1.269
11	17	7.45	92.3	30.32	942	63.5	23.51	826	48.0	7.40	3.06	0.639	0.727
12	17	7.26	52.2	22.80	905	42.3	16.42	806	42.7	5.85	3.98	0.789	0.730
13	17	7.27	94.8	16.39	927	81.0	12.60	829	37.5	6.14	4.76	0.840	0.754
14	17	7.45	106.8	28.85	918	76.8	22.50	792	49.9	8.80	3.17	0.665	0.725
	17	7.50	107.1	26.91	880	73.9	19.63	765	58.6	8.91	5.05	0.651	0.690
	21	7.43	107.6	25.24	891	72.7	18.40	768	54.3	8.53	6.03	0.652	0.704
	28	7.45	105.1	29.09	890	73.8	21.60	797	52.7	9.37	4.84	0.661	0.701

TABLE I—Continued

Case No.	Day of observation	pH	Plasma			Cells		Whole blood H ₂ O	Cell volume	O ₂ capacity per liter of blood	O ₂ content per liter of blood	$\frac{(\text{Cl})_c}{(\text{Cl})_p}$ Corrected to 100 per cent oxygenation	$\frac{(\text{HCO}_3)_c}{(\text{HCO}_3)_p}$ Corrected to 100 per cent oxygenation
			Cl	HCO ₃	H ₂ O	Cl	HCO ₃						
			mm per 1000 gm. H ₂ O	mm per 1000 gm. H ₂ O	gm. per l.	mm per 1000 gm. H ₂ O	mm per 1000 gm. H ₂ O	gm. per l.	per cent	mm	mm		
15	17	7.37	109.2	28.78	931	76.5	20.30	817	43.8	5.85	1.16	0.639	0.642
	14	7.38	112.6	26.61	940	87.3	21.20	863	36.4	6.02	2.03	0.726	0.747
	21	7.36	106.1	26.60	937	74.6	22.90	835	41.0	6.27	3.21	0.663	0.821
	69	7.43	113.8	24.91	930	82.2	18.79	860	36.9	6.66	1.78	0.661	0.693
	106	7.43	109.6	28.38	944	80.2	23.46	846	39.5	7.10	2.84	0.685	0.777
16	17	7.42	113.6	32.77	922	78.7	28.38	834	39.4	7.16	2.29	0.635	0.809
	7	7.48	112.7	32.56	935	71.8	23.08	844	37.7	6.78	2.45	0.581	0.653
	35	7.44	111.2	30.52	928	75.2	21.38	841	41.3	7.20	1.70	0.612	0.637
	80	7.48	101.3	29.84	948	68.8	22.21	824	46.0	7.70	4.59	0.643	0.708
	183	7.29	101.8	23.55	938	76.0	18.99	867	35.1	4.90	0.75	0.687	0.747
	194	7.38	104.6	21.34	931	77.8	20.18	836	33.8	5.01	1.12	0.682	0.885
17	17	7.56	99.3	33.13	949	68.1	24.50	839	38.0	6.52	2.58	0.627	0.682
	7	7.59	99.3	35.97	913	69.9	25.79	831	44.4	5.93	3.71	0.667	0.680
	29	7.54	97.5	32.34	928	63.6	24.30	848	35.5	6.00	3.96	0.620	0.719
18	17	7.48	105.9	31.89	916	68.9	23.46	808	45.9	8.46	2.62	0.590	0.675
	292	7.44	102.9	32.35	944	73.8	26.25	823	43.9	6.32	2.97	0.672	0.766
	299	7.49	106.0	30.66	934	73.5	22.32	823	43.7	7.23	4.44	0.660	0.694
	317	7.52	104.8	31.76	939	66.9	22.36	826	45.4	7.54	2.02	0.572	0.637
	343	7.52	93.6	31.98	941	68.8	25.07	772	55.7	9.92	6.71	0.705	0.754
	357	7.53	98.5	33.70	921	69.5	24.98	838	43.2	7.62	4.78	0.670	0.706
19	17	7.39	106.4	26.94	954	78.4	22.64	846	37.5	6.96	2.42	0.672	0.789
	35	7.41	90.4	36.88	936	63.6	29.43	871	36.8	7.72	4.60	0.671	0.791
20	17	7.54	91.3	39.23	934	62.5	32.38	864	35.9	5.54	2.09	0.626	0.769
	9	7.56	89.3	38.65	942	64.9	33.84	861	35.0	5.47	1.37	0.655	0.804
	17	7.54	90.0	36.69	933	62.4	27.98	866	35.5	5.09	2.86	0.652	0.742
	25	7.56	86.4	37.20	960	59.4	29.13	903	25.2	3.70	1.29	0.624	0.720
21	17	7.50	109.0	32.37	929	74.4	27.47	820	43.3	5.93	2.86	0.636	0.802
	11	7.51	109.6	32.82	942	79.0	29.06	805	44.0	6.59	2.16	0.661	0.824
	16	7.41	113.9	29.97	915	79.8	24.78	833	39.1	6.82	2.99	0.655	0.781
	69	7.52	109.6	32.43	925	71.9	25.84	836	39.8	7.53	2.53	0.595	0.736

TABLE I—*Concluded*

Case No.	Day of observation	pH	Plasma			Cells		Whole blood H ₂ O	Cell volume	O ₂ capacity per liter of blood	O ₂ content per liter of blood	$\frac{(\text{Cl})_c}{(\text{Cl})_p}$ Corrected to 100 per cent oxygenation	$\frac{(\text{HCO}_3)_c}{(\text{HCO}_3)_p}$ Corrected to 100 per cent oxygenation
			Cl	HCO ₃	H ₂ O	Cl	HCO ₃						
mM per 1000 gm. H ₂ O	mM per 1000 gm. H ₂ O	gm. per l.	mM per 1000 gm. H ₂ O	mM per 1000 gm. H ₂ O	gm. per l.	per cent	mM	mM					
22	17.45	102.8	30.64	939	73.4	25.39	825	42.0	7.33	3.15	0.665	0.780	
23	17.49	106.6	31.71	933	80.2	29.47	804	42.0	7.66	4.38	0.716	0.874	
24	17.46	106.5	31.55	934	81.3	26.45	799	56.4	10.06	3.12	0.704	0.779	
25	17.50	107.0	29.04	920	74.7	23.80	836	38.6	6.50	2.96	0.649	0.770	
26	17.50	107.4	31.05	933	80.3	28.36	837	38.4	5.90	1.96	0.687	0.853	
27	17.53	108.6	29.73	947	74.7	22.35	810	50.8	9.40	5.69	0.651	0.715	
28	17.55	102.8	31.81	928	63.0	22.12	826	48.2	8.19	6.02	0.589	0.670	
29	17.46	107.0	30.30	930	77.6	24.91	822	45.8	8.93	3.10	0.669	0.766	
30	17.37	105.6	29.03	942	80.7	23.46	827	40.8	7.25	3.38	0.723	0.767	
31	17.44	104.1	34.00	937	69.2	24.33	824	43.1	8.40	4.14	0.622	0.673	
32	17.43	109.1	30.89	921	73.2	22.66	813	51.3	8.78	3.39	0.620	0.683	
33	17.46	114.5	28.06	938	83.1	22.52	854	37.3	6.87	1.43	0.657	0.735	
34	17.41	108.2	28.61	919	81.1	22.41	815	45.2	7.10	6.16	0.738	0.772	
35	17.49	104.5	26.27	953	75.9	20.96	824	46.5	8.04	7.80	0.723	0.795	
36	17.43	109.6	24.99	946	75.5	23.79	797	40.3	5.90	2.30	0.650	0.915	
37	17.36	106.4	27.97	906	82.0	21.77	760	49.9	8.63	1.43	0.707	0.714	
38	17.44	106.9	29.77	922	78.9	23.84	829	47.1	7.98	3.22	0.687	0.751	
39	17.41	93.0	30.36	916	64.9	23.43	824	39.0	6.92	3.69	0.671	0.745	
40	17.39	110.5	23.11	922	75.1	19.80	833	46.5	7.54	2.87	0.633	0.808	
41	17.45	102.4	19.34	923	67.0	15.00	820	47.4	9.85	6.32	0.623	0.744	
42	17.40	109.1	19.47	921	80.9	15.50	833	43.4	7.52	4.15	0.706	0.761	
43	17.45	99.4	25.86	931	62.9	18.70	817	46.6	9.75	5.47	0.597	0.686	
44	17.44	111.4	25.25	933	79.8	18.89	845	35.9	6.75	2.18	0.642	0.693	
45	17.52	112.0	24.14	916	82.3	18.30	821	38.1	6.01	3.75	0.708	0.725	
46	17.43	121.6	17.84	922	72.1	13.05	812	37.6	6.70	4.61	0.568	0.702	
47	17.49	112.6	23.69	911	79.0	18.64	849	43.8	7.20	2.53	0.645	0.730	
48	17.48	111.4	23.05	924	69.6	16.83	857	33.2	5.58	3.68	0.595	0.700	
Max....	7.59	121.6	39.23					58.6			1.029	1.269	
Min....	7.07	52.2	6.89					15.7			0.568	0.637	
Aver....	7.42										0.669	0.764	

Summary of Cases

Chronic nephritis....	Cases 1-7	Severe hypertension. Cases 19-34
Bichloride poisoning. "	8-13	Alcoholic jaundice.. " 35-36
Nephrosis.....	" 14-15	Peritonitis..... Case 37
Cardiovascular renal disease.....	" 16-17	Obesity..... " 38
Arteriosclerosis.....	Case 18	Diabetes..... " 39
		Maternity..... Cases 40-48

At the bottom of Table I may be found the maximum, minimum, and average r values which had been corrected to 100 per cent blood oxygenation. The maximum and minimum values for the plasma pH, chloride, and bicarbonate, and cell volume are also

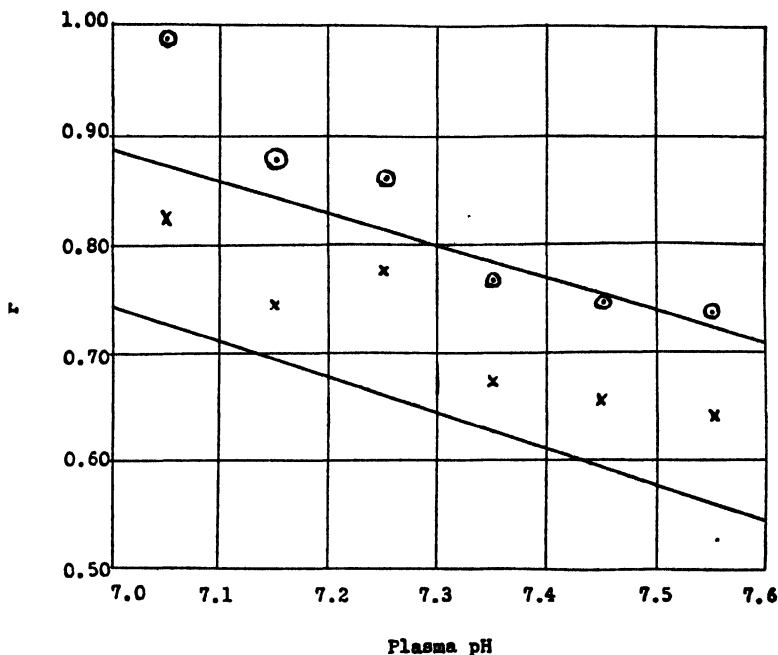


FIG. 1. Average values of chloride and bicarbonate distribution ratios in various pathological subjects corrected to 100 per cent blood oxygenation.

○ indicates average $r_{HCO_3} = \frac{\text{BHCO}_3 \text{ per kg. H}_2\text{O in cells}}{\text{BHCO}_3 \text{ per kg. H}_2\text{O in plasma}}$

× indicates average $r_{Cl} = \frac{\text{Cl per kg. H}_2\text{O in cells}}{\text{Cl per kg. H}_2\text{O in plasma}}$

The solid curves represent the mean values for the two ratios obtained by Van Slyke, Hastings, Murray, and Sendroy (3), oxygenated horse blood being employed. The upper curve represents the r_{HCO_3} .

given. The maximum variation in pH encountered was from 7.07 to 7.59, in plasma chloride from 52.2 to 121.6 mm per 1000 gm. of plasma water, in plasma bicarbonate from 6.89 to 39.23 mm per 1000 gm. of plasma water, and in cell volume from 15.7 to

58.6 per cent. When the data as a whole are considered, the r_{Cl} and r_{HCO_2} values appear quite constant in a given plasma pH range despite considerable variation in cell volume. Further, individual cases observed over considerable periods with little change in plasma pH also had uniform r_{Cl} and r_{HCO_2} ratios. This appears more true for the r_{Cl} than for the r_{HCO_2} . The maximum observed r_{Cl} of 1.029 was found with Case 10 suffering from bichloride poisoning, the plasma pH recorded, namely 7.07, being the lowest in the series. This case also gave the highest observed r_{HCO_2} of 1.269. The minimum r_{Cl} value of 0.568 was observed with Case 46, accompanied with a plasma pH of 7.43. The lowest r_{HCO_2} value of 0.637 was observed with Case 18 having a plasma pH of 7.52. The average r_{Cl} and r_{HCO_2} values were 0.669 and 0.764, respectively, with an average pH of 7.42. The ratio $r_{\text{Cl}} : r_{\text{HCO}_2}$ was 0.876.

The values for the distribution ratios were grouped according to pH and averaged. For example, values for the distribution ratios between 7.30 and 7.39 were grouped and averaged and the average point placed at 7.35 in the figure. Fig. 1 shows a plot of the average r_{Cl} and r_{HCO_2} values obtained at the various pH levels. As can be seen, the average points follow quite well the slope of the lines obtained by Van Slyke, Hastings, Murray, and Sendroy (3), oxygenated horse blood being employed. The r_{Cl} values appear to be definitely higher than the r_{Cl} values for horse blood, while the r_{HCO_2} values are somewhat closer to the r_{HCO_2} values for horse blood.

Discussion of Results

At the outset it must be acknowledged that determining the cell concentrations from whole blood and plasma analyses is open to criticism. In spite of this criticism, it is felt, since the distribution ratios reported were comparatively uniform and agree very well with previously published results, that reliable conclusions may be drawn.

Although a number of pathological conditions were examined, differing quite markedly in plasma pH, cell volume, and chloride and bicarbonate concentrations, the distribution ratios are quite similar. Hastings, Sendroy, McIntosh, and Van Slyke (4) obtained on nine normal subjects with an average pH, of 7.38 average

$\frac{(\text{Cl})_c}{(\text{Cl})_p}$ and $\frac{(\text{BHCO}_3)_c}{(\text{BHCO}_3)_p}$ ratios of 0.689 and 0.786, respectively. This agrees very well with the average values of this series; namely, $\frac{(\text{Cl})_c}{(\text{Cl})_p} = 0.669$ and $\frac{(\text{BHCO}_3)_c}{(\text{BHCO}_3)_p} = 0.764$ at an average plasma pH of 7.42. The average $r_{\text{Cl}} : r_{\text{HCO}_3}$ of 0.876 also agrees very well with the average figure found by Hastings *et al.*, namely 0.87.

As can be seen from Fig. 1, which presents the average distribution ratios corrected to 100 per cent blood oxygenation for a given pH range, both the r_{Cl} and r_{HCO_3} decrease as the plasma pH increases. Further, the average values parallel quite closely the lines in the figure which represent the mean values for the two ratios obtained by Van Slyke, Hastings, Murray, and Sendroy (3), oxygenated horse blood being employed. The r_{Cl} is definitely above the mean value for horse blood in each case, while the r_{HCO_3} values approach those for horse blood more closely. Due to the necessity for economy of material the colorimetric pH was employed in all these determinations, although more uniform results would probably have been obtained had the hydrogen electrode been used for these determinations. With each ratio a value above unity was obtained. In Case 10 with bichloride poisoning and a plasma pH of 7.07, a r_{Cl} value of 1.029 and a r_{HCO_3} value of 1.269 were obtained. Henderson, Bock, Dill, Hurxthal, and van Caulaert (10) report high distribution ratios in the blood of a case of terminal chronic nephritis. They found values of r_{Cl} and r_{HCO_3} of 0.903 and 1.33, respectively, for arterial blood.

Christie, Sendroy, and Van Slyke (11) have studied the distribution of electrolytes in hemophilic blood after equilibration with CO_2 tensions varying between 25 and 197 mm. and found a normal shift in Cl. In this laboratory we have been studying the chloride and bicarbonate distribution ratios in bloods obtained from various pathological subjects at equilibrium after equilibration with varying tensions of CO_2 . Although these data will be reported in the near future it may be stated now that normal distribution ratios, which vary as predicted, have been found in the various conditions studied to date. The results of Harkins and Hastings (12) are of interest in this connection. These authors made a study of the

electrolyte equilibrium in the blood in experimental acidosis in dogs produced by injecting large amounts of hydrochloric acid with the conclusion that the physicochemical laws, found to be adequate in predicting the distribution of diffusible ions between cells and serum *in vitro*, are also adequate to account for their distribution in acute experimental acidosis *in vivo*.

The data as a whole lend support to the conclusion of Hastings, Sendroy, McIntosh, and Van Slyke (4) that the r variations at a given plasma pH show no greater variation in pathological conditions than in normals presumably because of the constancy of the hemoglobin concentration in the cells and the total base of the serum; these two factors being the most important in fixing the value of r . Cameron and Foster (13) made the interesting observation that in pernicious anemia the hemoglobin content of a given volume of red blood corpuscles is invariably above the average normal value while the chloride content is always below the average normal value. This is not surprising since it is an old observation that the color index is increased in pernicious anemia. Consequently, at a given plasma pH an increased concentration of hemoglobin in the cells would necessitate a lowered cell chloride.

CONCLUSIONS

The distribution of chloride and bicarbonate between plasma and cells has been studied in a number of pathological conditions.

The distribution ratios at a given plasma pH are quite similar irrespective of the pathological condition.

The average distribution ratios in a given pH range when plotted against pH vary in accord with the conclusions outlined by Van Slyke, Wu, and McLean.

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A COMPARISON OF THE CHLORIDE AND BICARBONATE CONCENTRATIONS BETWEEN PLASMA AND SPINAL FLUID AND PLASMA AND ASCITIC FLUID IN REFERENCE TO THE DONNAN EQUILIBRIUM

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Van Slyke and his coworkers (1-4) have discussed the factors affecting the electrolyte and water distribution between serum and corpuscles and between serum and transudates with the development of equations according to the Gibbs-Donnan law predicting the influence of these factors on the distribution. An unequal distribution of electrolytes between serum and spinal fluid or between serum and ascitic fluid may be expected since the serum contains a great deal more protein than either of the other two fluids. As a consequence Van Slyke, Wu, and McLean (1) predicted the following equation,

$$(1) \quad r_{sf} = \frac{[BP]_f + \sqrt{[BP]_f^2 + 4[A]_s \cdot ([A]_s + [BP]_s)}}{2([A]_s + [BP]_s)}$$

to give the Donnan distribution ratio between serum and transudate, in which [BP] and [A] represent, in molal concentration, the base bound by protein and anions other than protein, respectively. The subscripts *s* and *f* represent serum and fluid. If the protein concentration of the transudate is considered negligible the equation becomes simplified to

$$(2) \quad r_{sf} = \sqrt{\frac{(A)_s}{(A)_s + (BP)_s}}$$

With the comparison of the electrolyte concentration of the serum and serous cavity fluids, Loeb, Atchley, and Palmer (5)

concluded that the relationship between serum and edema fluid seems to result from a simple membrane equilibrium, influenced in part by the proteins present. These authors found that when the edema fluid of a patient was placed in a collodion sac and dialyzed against serum of the same subject, no change in the electrolyte concentration occurred indicating that the two fluids were in approximate equilibrium *in vivo*. Van Slyke, Wu, and McLean (1) found, except in the case of the chloride distribution, that there was considerable discrepancy between the ratios calculated from the data of Loeb, Atchley, and Palmer and those predicted by formula. Hastings, Salvesen, Sendroy, and Van Slyke (4) made further comparison of the distribution ratios between serum and edema fluid which were found by analysis and those calculated from theory with the conclusion that the ratios of HCO_3 , Cl, Na, and H^+ deviate from unity as predicted.

Just as the present paper was being prepared for publication the report of Greene, Bollman, Keith, and Wakefield (6) appeared. These authors studied the distribution ratios of the various anions and cations between serum and transudate in experimentally produced edema or ascites in dogs and in hospital patients where samples of ascitic or pleural fluid were obtained by paracentesis. The results obtained seem to give further evidence that the distribution of the various electrolytes between blood serum and transudate is governed by physicochemical laws.

Hamilton (7) compared the concentration of inorganic substances in serum and spinal fluid and found that although indications are not lacking that an equilibrium of the Donnan type at least partly governs the distribution of electrolytes, it seemed to him probable that the equilibrium is modified by unknown factors. This conclusion was arrived at since considerable variation of the HCO_3 ratio and to a lesser extent variation of the Cl ratio occurred, although the ratio of the sum of HCO_3 and Cl was more constant. Linder and Carmichael (8) considered the distribution ratios of the Cl and HCO_3 between serum and spinal fluid in meningitis, in which there was a decreased chloride concentration of the spinal fluid. It was found that the Cl ratio was markedly constant and hence excludes the possibility of an altered permeability as the cause of the decreased spinal fluid chloride. The constant ratio was given as an additional fact supporting the

view that the Donnan law has an important rôle in determining the composition of the fluid.

Pincus and Kramer (9) also concluded from their study of the concentration of the various cations and anions in spinal fluid and serum that the Donnan membrane equilibrium plays an important part.

The present report contains further data on the distribution of Cl and HCO_3 between plasma and spinal fluid and plasma and ascitic fluid. The plasma, spinal fluid comparisons were made upon various hospital cases while the plasma, ascitic fluid comparisons were made upon three cases of nephrosis with massive edema.

Methods

The blood was drawn before breakfast from an arm vein under oil, without stasis, and treated as previously described (10). Immediately after drawing the blood sample the spinal fluid or ascitic fluid was collected and preserved under oil. None of the reported samples of spinal or ascitic fluids was contaminated with blood.

The pH was determined colorimetrically according to the procedure of Myers and Muntwyler (11). A C correction of 0.15 pH was employed for the spinal fluid. The spinal fluid pressure, where recorded, was determined by employing an Ayer manometer. All the other determinations were performed by the methods previously discussed (10).

Calculations

The bicarbonate concentration of the serum was determined by the following formula.

$$\text{mm BHC}_3 = \text{CO}_2 - \frac{\text{CO}_2}{10^{\text{pH} - 6.10} + 1}$$

in which CO_2 is the total CO_2 determined in mm concentration. The bicarbonate concentration of both the ascitic fluid and spinal fluid was determined with the same formula with the exception that a value of 6.13 instead of 6.10 was employed. A pH of 7.40 was assumed for the ascitic fluid in every case. The mm con-

TABLE I
Comparison of Chloride and Bicarbonate Concentrations in Spinal Fluid and Plasma

Case	Date	Sample	pH	Base mM per l.	H ₂ O gm. per 100 cc.	Cl mM per 1000 gm. H ₂ O	HCO ₃ mM per 1000 gm. H ₂ O	Protein mM per 1000 gm. H ₂ O	Pressure	$r = \frac{(\text{Cl})^p}{(\text{Cl})^{s.f.}}$	$r = \frac{(\text{HCO}_3)^p}{(\text{HCO}_3)^{s.f.}}$	$r = \frac{(\text{Cl})^p + (\text{HCO}_3)^p}{(\text{Cl})^{s.f.} + (\text{HCO}_3)^{s.f.}}$	r calculated
T. W., hypertension	Sept. 12, 1928	Plasma Fluid	7.45	145.5	942	102.3	30.0	17.4		0.846	1.28	0.917	0.940
			7.44	145.2	990	120.9	23.4						
J. W., hypertension	" 19, 1928	Plasma Fluid	7.39	155.5	954	106.4	26.9	14.5	24.5	0.854	1.19	0.906	0.950
			7.29		997	124.6	22.6						
	Oct. 9, 1928	Plasma Fluid	7.52	142.2	937	99.2	30.6	16.7	30.0	0.825	1.18	0.887	0.941
			7.51		992	120.3	26.0						
J. H., hypertension	" 24, 1928	Plasma Fluid	7.41	140.0	936	90.4	36.9	16.1	42.0	0.824	1.29	0.920	0.942
			7.29	152.2	998	109.7	28.6						
	Mar. 22, 1929	Plasma Fluid	7.50	148.8	927	113.0	30.0	17.5	21.0	0.884	1.23	0.939	0.944
				155.5	995	127.8	24.4						
J. U., nephrosis	June 22, 1929	Plasma Fluid	7.50	156.8	928	105.2	32.9		16.5	0.831	1.27	0.906	
			7.41	165.1	998	126.6	25.9						
	Oct. 17, 1928	Plasma Fluid	7.35	145.8	946	109.3	28.2		17.0	0.879	1.20	0.930	
			7.39		998	124.4	23.5						
	" 31, 1928	Plasma Fluid	7.30	146.5	926	119.8	20.9	10.5	17.0	0.922	1.07	0.941	0.965
			7.41	154.0	998	129.9	19.6						

P. K., nephrosis	Apr. 1, 1929	Plasma	7.49		917	107.3	26.8	15.7	11.5	0.896	1.11	0.909	0.946
		Fluid	7.41	151.4	998	123.5	24.1						
	" 19, 1929	Plasma	7.50		905	109.2	25.3	14.3	18.0	0.857	1.16	0.900	0.950
		Fluid	7.48		979	127.7	21.8						
S. B., nephritis	Mar. 15, 1929	Plasma	7.45		933	114.2	25.8		20.0	0.901	1.12	0.934	
		Fluid	7.48		997	126.8	23.0						
H. M., bichloride poisoning	Oct. 16, 1928	Plasma	7.31	132.1	933	90.5	15.9	18.9	16.0	0.744	0.77	0.748	0.921
		Fluid	7.43	147.9	996	121.6	20.7						
F. T., bichloride poisoning	Sept. 18, 1928	Plasma	7.45	147.0	942	92.3	30.4	18.3	11.5	0.803	1.43	0.900	0.933
		Fluid	7.32	152.1	997	115.0	21.3						
M. F., bichloride poisoning	May 16, 1929	Plasma	7.22	134.0	928	94.7	15.3			0.789	0.85	0.797	
		Fluid	7.37	161.0	999	120.1	18.0						
M. L., chronic nephritis	Sept. 14, 1928	Plasma	7.44	146.6	929	99.0	29.5			0.826	1.29	0.901	
		Fluid	7.36	149.0	989	119.8	22.9						
L. S., hypertension	Aug. 3, 1929	Plasma	7.37	166.0	919	109.8	25.2	20.3	10.0	0.871	1.02	0.896	0.932
		Fluid	7.44	179.0	998	126.1	24.6						
	Sept. 8, 1929	Plasma	7.41	160.2	916	93.0	30.3	20.8	12.5	0.786	1.16	0.854	0.925
		Fluid	7.50	177.0	999	118.3	26.1						
M. J., chronic nephritis	Nov. 6, 1929	Plasma	7.37	160.2	930	116.3	15.2	17.9	22.5	0.897	1.09	0.915	0.938
		Fluid	7.51	172.0	990	129.7	14.0						
Average.....													0.858
Maximum.....													0.910
Minimum.....													0.941
													0.786
													1.07
													0.854

The summary does not include the bichloride poisoning cases.

738 Chloride and Bicarbonate Distribution

centrations per 1000 gm. of water were obtained from the concentrations per 1000 cc. and the gm. of water per 1000 cc.

The distribution ratio " r calculated" given in the tables was obtained with the aid of equation (2). The concentrations in this equation are expressed in mm per 1000 gm. of water; (A), being taken as the sum of the Cl and HCO_3 concentrations in the plasma and (BP), the base bound by protein in the plasma. The latter was estimated with the aid of the formula given by Peters, Wakeman, Eisenman, and Lee (12),

$$\text{BP} = 1.072 \text{ P}(\text{pH} - 5.04)$$

where BP and P represent base combined with protein in mm per liter concentration and per cent protein respectively.

Results

Cl and HCO_3 in Plasma and Spinal Fluid

The results of the comparison of the chloride and bicarbonate concentrations of the plasma and spinal fluid are collected in Table I. Table II contains the comparison of the plasma and ascitic fluid. At the bottom of each table are given the maximum, minimum, and average r values obtained. In Table I Cases H. M., F. T., and M. F. were not included in the average inasmuch as the bichloride poisoning causes the concentrations of plasma chloride and bicarbonate to change more rapidly than the spinal fluid and the figures therefore do not represent equilibrium conditions.

If the bichloride poisoning cases are excluded from the discussion, it is observed that all the $r = \frac{(\text{HCO}_3)_p}{(\text{HCO}_3)_{s.f.}}$ are above unity, the average being 1.18 with variations between 1.29 and 1.07. The $r = \frac{(\text{Cl})_p}{(\text{Cl})_{s.f.}}$ was found to average 0.858 with variations between 0.922 and 0.786, and the $r = \frac{(\text{Cl})_p + (\text{HCO}_3)_p}{(\text{Cl})_{s.f.} + (\text{HCO}_3)_{s.f.}}$ to average 0.910 with variations between 0.941 and 0.854. The average calculated r was 0.943.

Cl and HCO₃ in Plasma and Ascitic Fluid

As with the spinal fluid comparisons, with one exception, the $r = \frac{(\text{HCO}_3)_p}{(\text{HCO}_3)_{a.f.}}$ was greater than unity, averaging 1.076 and varying between 1.16 and 0.979. The $r = \frac{(\text{Cl})_p}{(\text{Cl})_{a.f.}}$ values in contrast to the spinal fluid, were closer to unity, averaging 0.959 and varying between 0.975 and 0.940. The values for the $r = \frac{(\text{Cl})_p + (\text{HCO}_3)_p}{(\text{Cl})_{a.f.} + (\text{HCO}_3)_{a.f.}}$ were quite uniform to average 0.978.

Discussion of Results

At the outset it should be pointed out that the distribution ratios for plasma and spinal fluid were obtained from analyses on venous blood plasma and that conditions more representative of equilibrium probably would be obtained by employing arterial blood. By employing venous blood plasma the bicarbonate ratio is augmented while the chloride ratio is decreased since arterial blood plasma contains less bicarbonate and more chloride than the venous. However, even though venous plasma figures are employed any abnormal distribution ratios should be detected.

The data for the plasma and spinal fluid chloride and bicarbonate concentrations are in accordance with the findings of both Hamilton (7) and Linder and Carmichael (8) where in both instances bicarbonate ratios above unity and chloride ratios below unity were observed. The chloride and the chloride plus bicarbonate ratios remained quite constant regardless of the type of pathological condition provided the changes of the plasma concentrations were not too rapid. Case J. W. showed a progressive decrease in the plasma chloride and increase in bicarbonate. The spinal fluid also showed an increase in bicarbonate and decrease in chloride. Apparently, however, some time is necessary to establish equilibrium since the chloride ratio showed a progressive decrease and the bicarbonate ratio an increase, while the calculated ratio remained practically unchanged. Case J. U. was given ammonium chloride between the two experimental observations. As a result of the ammonium chloride ingestion the plasma chloride had increased 10.5 mm while the plasma bicarbonate had decreased 7.3 mm. The spinal fluid coincidentally

showed an increase of 5.4 mm in chloride and a decrease of 3.9 mm in bicarbonate. Case L. S. showed similar changes. The spinal fluid pressure was determined in most instances and from the limited data there was no apparent relationship between the distribution ratios and the spinal fluid pressure level.

The plasma and ascitic fluid comparisons were made upon three cases of nephrosis with massive edema. These distribution ratios appear more constant than the spinal fluid comparisons and are in closer agreement to the calculated ratio. The bicarbonate ratio is, with one exception, greater than unity. No explanation is apparent for this finding which is not in accord with the bicarbonate ratios reported by Hastings, Salvesen, Sendroy, and Van Slyke (4) who found close agreement between the bicarbonate ratios and the calculated values. Greene, Bollman, Keith, and Wakefield (6) found an average of 1.033 for the ratio CO_2 in serum: CO_2 in fluid in their ten hospital cases, with three of the ten values being below unity. The chloride ratios and the ratios of the sum of the chloride and bicarbonate are quite close to the calculated ratios. The latter figure should undoubtedly be closer to unity since the protein concentration of the ascitic fluid was considered negligible. The chloride ratios reported here agree well with those of the above two groups of workers. The ratio anions in the serum: anions in the fluid obtained on the ten cases by Greene and his coworkers is 0.9798 while the ratio of
$$\frac{(\text{Cl})_p + (\text{HCO}_3)_p}{(\text{Cl})_{a.f.} + (\text{HCO}_3)_{a.f.}}$$
 obtained in these cases is 0.978.

Case J. U. received ammonium chloride between the two determinations. There was an increase in the plasma chloride of 10.5 mm and a decrease of 7.3 mm in the bicarbonate concentration. At the same time there was an increase of 13.4 mm in ascitic fluid chloride and 8.8 mm decrease in bicarbonate.

Cases J. U. and P. K. were examined at the height of edema formation, while Case J. D. was examined when edema was rapidly appearing (first determination) and also when the edema was disappearing (last determination). From observation of the distribution ratios it is apparent that no special permeability of the membrane exists in which a piling up of chloride in the ascitic fluid should occur. Further, there is no apparent difference in the distribution ratios at the height, during increase, or during the disappearance of the edema to explain either its development or

reabsorption. The results strongly suggest that the concentration and distribution of electrolytes in the body fluids are governed by the factors outlined for the Donnan equilibrium.

CONCLUSIONS

1. A study of the chloride and bicarbonate distributions between plasma (venous) and spinal fluid and plasma and ascitic fluid has been made.

2. The average values of $r = \frac{(\text{Cl})_p}{(\text{Cl})_{s.f.}} = 0.858$, $r = \frac{(\text{HCO}_3)_p}{(\text{HCO}_3)_{s.f.}} = 1.18$, and $r = \frac{(\text{Cl})_p + (\text{HCO}_3)_p}{(\text{Cl})_{s.f.} + (\text{HCO}_3)_{s.f.}} = 0.910$ were obtained for spinal fluid.

3. The average values of $r = \frac{(\text{Cl})_p}{(\text{Cl})_{a.f.}} = 0.959$, $r = \frac{(\text{HCO}_3)_p}{(\text{HCO}_3)_{a.f.}} = 1.076$, and $r = \frac{(\text{Cl})_p + (\text{HCO}_3)_p}{(\text{Cl})_{a.f.} + (\text{HCO}_3)_{a.f.}} = 0.978$ were obtained for ascitic fluid.

4. The results of this study strongly suggest that the concentration and distribution of electrolytes in the body fluids are governed by the factors outlined for the Donnan equilibrium.

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A REPORT ON THE EFFECT OF LIGHT ON PIGMENT FORMATION IN APPLES

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INTRODUCTION

Investigations have been carried out in recent years in an effort to improve the color of various fruits. Citrus and other semi-tropical fruits, in particular, have received the most attention. Several studies of the factors influencing the formation of the red pigment of apples have been made. In general, such studies have included the influence of light, fertilization, pruning, and artificial tree and fruit feeding.

Overholser (1) has shown that light is one of the most important factors in the development of anthocyanin pigments, not only in the apple, but in other kinds of fruit as well, such as pears, peaches, plums, nectarines, etc.

Magness (2) has presented data which indicate that apples must reach a certain state of maturity before red color will develop on exposure to light. In other words, certain substances must be present in the fruit before the anthocyanin pigments can be produced by the action of light.

Since light is such an important factor in anthocyanin formation, it becomes essential to determine which wave-lengths of light are most influential. The present paper is a preliminary report on the effect of various wave-lengths of light on development of color in McIntosh apples.

Before going further, it is desirable to establish an arbitrary range for the main divisions of the spectrum. According to Luckiesh (3) the ultra-violet range is from 0 to 3000 Å., the near ultra-violet from 3000 to 4000 Å., and the visible spectra from 4000 to 7600 Å.

Some Common Observations on Pigment Formation

The following discussion deals primarily with observations made on McIntosh apples. In the absence of light, mature green apples will lose their green color and become yellowish. This process is no doubt nothing more than the disappearance of chlorophyll with the consequent appearance of the yellow pigments which were masked by the green color. Similar observations have been reported by Overholser (1), Magness (2), and Fletcher (4) in regard to other varieties of apples which develop red pigments.

Apples washed in dilute HCl or exposed to the fumes seem to develop a small amount of red color.¹ They become quite yellow with streaks of red. The red pigmentation is probably due to the fact that the anthocyanins are oxonium salts of either plant or mineral acids. In this case, HCl is readily available. However, the use of this acid seems to have slight possibilities for producing intensive pigmentation.

Experience has also taught that mature apples will develop anthocyanins when exposed to sunlight no matter whether they are green or have changed to yellow. Under poor light conditions they may go through the three stages of green, yellow, and red. It has been noticed that those parts of the apple which have been shaded by leaves or spray deposits are never colored red, but on exposure to sunlight the red pigment is developed. This indicates that neither the parent substance nor the final red pigment is readily diffusible between the various cells.

EXPERIMENTAL

McIntosh apples were used throughout the experiments. The apples selected for use were mature but had only traces of red color development.

The amount of color was estimated macroscopically on the basis of the percentage of the apple surface colored.

In the experiment in which an ultra-violet lamp was used as a light source, no filters were employed.

¹ W. H. Harman and L. R. Streeter observed that in some cases McIntosh apples washed with HCl to remove spray residue showed increased color development.

Five lots of apples were exposed twice daily for 9 days to the rays of a mercury vapor lamp (Alpine sun) placed 30 inches away, in the following manner:

	<i>min.</i>
Lot 1.....	1
" 2.....	2
" 3.....	3
" 4.....	4
" 5.....	5

The apples were kept in the dark in an ice refrigerator held at 10°.

No definite coloration was detected in any lot and severe injury to the flesh and skin of the apples resulted. The fruit became pitted and was discolored at the points of indentation.

Experiments described by Magness (2) in which he compared coloration under window glass and in direct sunlight led him to suspect that the ultra-violet region is the important part of the spectrum. He found that the apples under window glass did not color as extensively as they did in direct sunlight. He was also able to color Jonathan apples by exposure to an ultra-violet lamp. Our results are not quite in accord with those of Magness, and no explanation for the disagreement is offered because we are not familiar with the details of his experiments.

Effect of Various Portions of the Solar Spectrum

Wooden boxes were constructed with open back, front, and bottom. Wire screening was stretched over the bottom and various light filters placed in the front, while black cloth was stretched over the back. The apples were then placed in the boxes and the whole placed over a pan of ice and set in a position such that the apples would receive a maximum amount of sunlight.

In these experiments colored glass light filters were employed which were designed to transmit only certain parts of the spectrum. Table I gives a brief summary of the results.

It will be noted that some coloration was obtained with all the filters, except the heat-transmitting glass. This is no doubt due to reflected light from the ice and possibly to the exposure to direct sunlight during the frequent examinations of the fruit.

Fig. 1 brings out more clearly the influence of the various portions of the spectrum. Filters 403, 585, Pyrex, and window glass produced a large amount of color, while those that produced practically no pigmentation were Filters 334, 224, 491, and H.T. From Fig. 1 it is observed that those glasses under which coloration developed have a transmission range of from 3000 to 7000+ Å. Those under which no color developed have a range of from 4400 to 7000+ Å. This indicates that the effective range is below 4400 Å. But it has been assumed that the reason for no

TABLE I

Effect of Light of Different Wave-Lengths on Color Development

Glass filters used		Range of transmission	Coloration in 7 days†
Code No.*	Description		
		Å.	per cent
Check	Direct sunlight		85
"	Window glass	3200-7000+	70
"	Pyrex glass	3100-7000+	70
H. T. check	Opaque heat-transmitting glass	Infra red	2
403	Blue-green, medium shade	3500-6000	50
585	Ultra-violet-transmitting blue-purple	3000-4800	50
224	Red	6200-7000+	5
334	Heat-resisting yellow special shade	5400-7000+	10
491	Sextant green	4400-6800	3

* Code Nos. are those of the Corning Glass Works, makers of the colored glass filters.

† Apples had from 1 to 4 per cent coloration before exposure.

pigment formation in the case of glass Filter 491 is the absence of the effective wave-lengths of light. This may not be the case at all because the curve for this glass in Fig. 1 shows only 50 per cent transmission at its maximum point while the region from 4300 to 4800 Å. is transmitted much less intensively. This would place the upper limit of the effective range at 4800 Å. It now remains to establish a lower limit, and it is readily seen to be at approximately 3200 Å. because both the Pyrex and window glass produced good coloration.

This range may be still further limited by a comparison of the two Filters 403 and 585. Table I shows that both filters produced the same amount of color. From a study of their transmission curves in Fig. 1 it is noted that they overlap each other. Since both produced the same extent of color, it is evident that the point

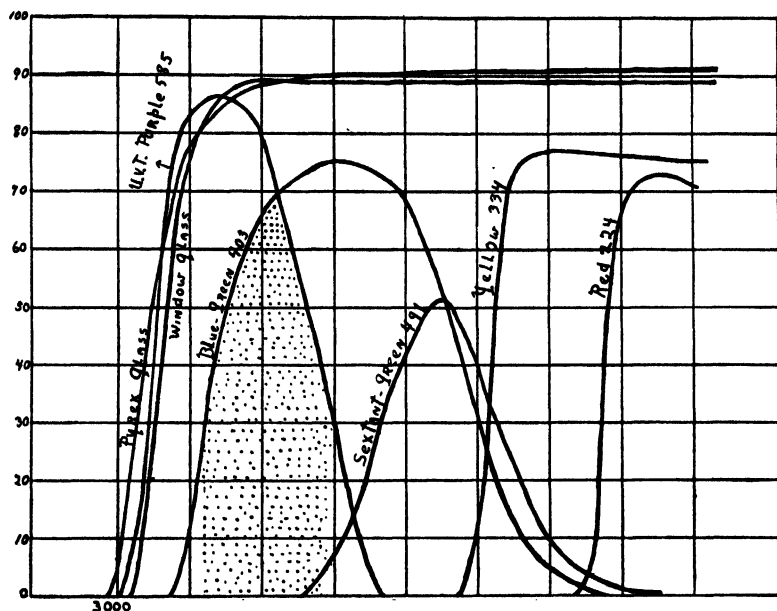


FIG. 1. Transmission of light by the filters employed. The per cent of transmission (ordinate) is plotted against the wave-length in Ångström units (abscissa). All curves except the one marked "window glass" were reproduced by permission of Director G. K. Burgess of the Bureau of Standards from Gibson, K. S., Tyndall, E. P. T., and McNicholas, H. J., *Bureau of Standards Tech. Paper No. 148* (1920). The curve for window glass was reproduced by permission of M. Luckiesh from his book "Artificial sunlight," (3) p. 110, Fig. 25.

at which their transmission curves intersect marks the approximate wave-length of most influence. Likewise, the region bounded by the intercepts of the two curves marks the most influential range of the spectrum. This region is shown as the dotted section of Fig. 1. Thus, we have established a range of from 3600 to 4500

Å., with an optimum at 4100 Å., as the most effective part of the solar spectrum on anthocyanin development in the McIntosh apple.

It is obvious that Pyrex and window glass will not interfere with color formation because both transmit this range completely and more intensively than any other glass employed. This accounts for the fact that the most color was produced under them.

Perkin and Everest (5) and Wheldale (6) have cited researches which show that quercetin on reduction will produce, as a by-product, a substance identical with a natural anthocyanin. This suggests that the flavonols may be the precursors of the anthocyanin pigments, but how this change is brought about *in vivo* is not known.

Shibata and Kimotsuki (7) and Tasaki (8), have studied the absorption spectra of a number of the flavones and flavonols. It is important to observe that the majority of the compounds they studied have a maximum absorption in the vicinity of 4000 Å. It would seem rather more than coincidence that we have found a region of the solar spectrum in the same vicinity which is most influential in coloring McIntosh apples. This indicates that the influence of light is through a flavonol from which the anthocyanin is derived. Sando (9) isolated and identified quercetin from McIntosh apple skins. Thus, it is established that a flavonol is present in McIntosh apples. Data on the absorption by the glucoside of quercetin (7) show that the introduction of sugar into the molecule does not alter the point of maximum absorption. It is evident that our results would support the postulate that the anthocyanin is formed from the flavonol or its glucoside, since the absorption by quercetin and quercetrin is practically the same in that part of the spectrum which we have shown to be most effective in producing pigmentation.

SUMMARY

1. The region 3600 to 4500 Å. of the solar spectrum has been shown to be most influential in coloring McIntosh apples.

2. It has been suggested that a flavonol is the substance which is transformed by the light energy into the red pigment.

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THE ACTION OF NEUTRAL SALTS ON THE OPTICAL ACTIVITY OF GELATIN

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(Received for publication, June 15, 1931)

The present paper offers an explanation for the previously noted effect (1) that a neutral salt increases the hydrogen ion concentration of a gelatin solution. Data are given to show that a neutral salt (added to a solution of electrolyte-free gelatin) decreases the optical activity of a gelatin solution while small amounts of HCl do not. The proposed explanation for this combined effect is that a neutral salt displaces the keto-enol equilibrium of a protein toward the enol side producing simultaneously an increase in hydrogen ion concentration and a decrease in optical activity.

As a result of the classic studies of Loeb (2), it is a well known fact that the action of neutral salts on solutions of gelatin salts can be accounted for on the basis of Donnan's theory of membrane equilibria and any changes due to salts can be predicted and expressed by a simple algebraic equation. Loeb found that sodium chloride added to gelatin chloride at a pH of 3.0 has no effect at all on the hydrogen ion concentration of such a solution.

There have been various theories advanced regarding the action of neutral salts on proteins. The most generally accepted is that introduced by Hardy (3) in his studies on the action of neutral salts, acids, and bases on globulin. He suggests that a neutral salt adds itself to an amino group of the protein molecule thus satisfying the unsaturated condition of the nitrogen. The action of neutral salts on proteins is reviewed by Lloyd (4).

Changes in hydrogen ion concentration, or in the optical rotation, as an action of neutral salts have not been reported (so far as is known) except by the author. Pauli, Samec, and Strauss (5), on the contrary, have found that pure neutral salts of the alkalis and alkaline earth do not influence the optical rotation of proteins.

It is well known that proteins can be enolized by the action of alkalis. The enolization of proteins by alkalis was first postulated by Dakin (6) who represented the change in optical activity

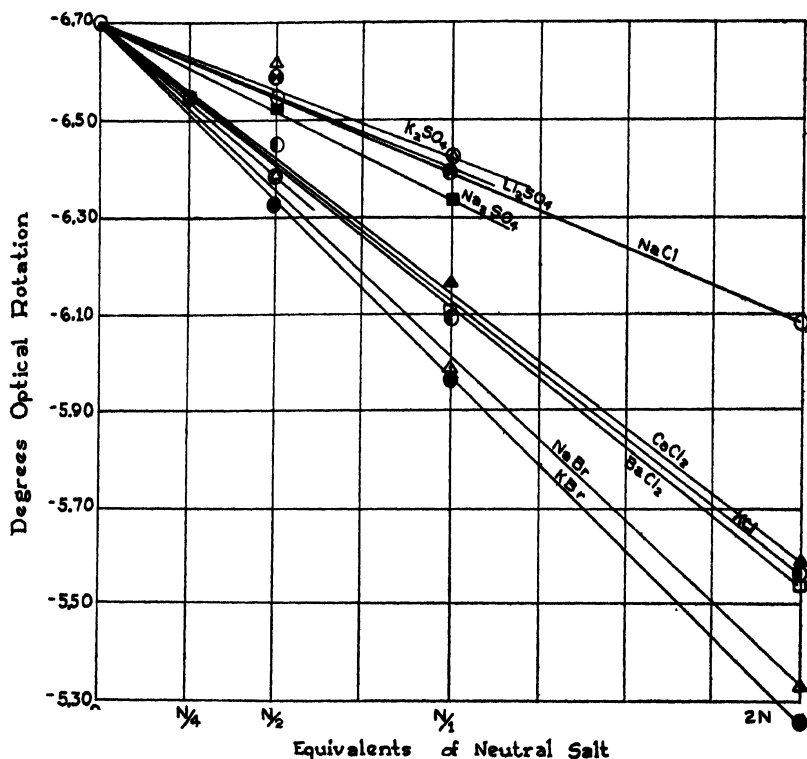


FIG. 1. Shows the action of various amounts of different neutral salts on the optical activity of a 2 per cent gelatin solution. Concentrations of salt greater than those indicated caused the precipitation of the gelatin.

Equivalents of neutral salt are arbitrarily chosen as the abscissa. No attempt at this time is made to generalize as to the action of the different salts. Data obtained with sulfates are generally less accurate than those obtained with chlorides.

to be due to a keto-enol tautomeric change taking place in an alkaline solution. (A review of enolization has been compiled by Graenacher (7).)

EXPERIMENTAL

The present experiments for determining the actions of neutral salts on the optical activity of gelatin were carried out with completely deashed gelatin. The method used in purifying this material has been described previously (8). The determinations of optical activity were carried out at 34.7° by means of a Schmidt and Haensch polarimeter (No. 10749). The source of light was a Cooper Hewitt mercury vapor lamp (Type 3552) with a Corning yellow light filter (heat-resisting yellow) and a Corning Didymius filter 5.54 mm. thick, giving a mean wave-length of about 5461 Ångström units. 2 per cent solutions of gelatin containing the desired amount of salt were placed in a water-jacketed polarimeter tube. Water was drawn through the cooling jacket of the polarimeter tube from a water bath a few inches distant, by means of a high speed rotary pump. The water bath was kept at 35.1° and did not vary more than a few hundredths of a degree. Consequently no appreciable variation could be observed in the temperature of the solution within the polarimeter tube. The data of Fig. 1 are representative of a number of repeated series of determinations made with different salts. Higher salt concentrations than these indicated resulted in the precipitation of gelatin or the production of a turbidity too great to make readings possible. The average of five readings was taken in each determination. Readings rarely varied more than 0.02° from the average obtained. The readings did not vary with time as they do in case of readings taken below 30° and appeared to come to an immediate equilibrium. 1 cc. of 0.1 N HCl added to 100 cc. of 2 per cent gelatin produced no noticeable change in its optical activity.

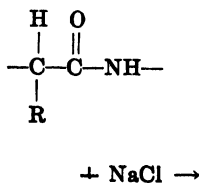
DISCUSSION

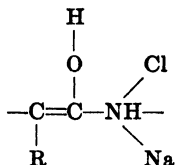
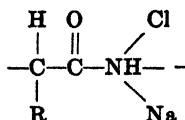
The action of a neutral salt on electrolyte-free gelatin cannot be explained on the basis of membrane equilibrium because there can be no common ion effect.

While the racemization of proteins by alkali appears to be a permanent one, the enolization by a neutral salt as in the present experiments, is assumed to consist of a tautomeric displacement which involves an equilibrium between the keto and the enol forms of the proteins and is proportionate to the salt concentration.

There is further evidence to support the probability that enolization plays a part in the behavior of proteins. Solutions of pure gelatin when cooled become opalescent. After the formation of a gel, this opalescence becomes more intense with time as well as with a decrease in temperature until it has assumed the intensity of the turbidity of milk. This opalescence decreases when the gel is warmed and disappears upon the melting of the gel. This opalescence also becomes less intense upon the addition of a neutral salt and disappears entirely if sufficient salt is added. That these phenomena are accompanied by a change in the keto-enol equilibrium of the protein is indicated by the experiments of Trunkel (9), who has found that the optical rotation of gelatin solutions decreases as the temperature is increased; that the optical rotation reaches an immediate equilibrium at 30° or above, but comes to a slow equilibrium below this temperature, and requires a longer time for a condition of equilibrium to be reached when the temperature is lowered. Gel formation is therefore accompanied by increased optical rotation which is possibly explained to be a consequence of decreased enolization.

If the addition of a neutral salt to a gelatin solution caused only an increase in hydrogen ion concentration without changing the optical activity, the more logical conclusion would be that neutral salts alter the hydrogen ion activity of the gelatin. The fact that the change in hydrogen ion concentration caused by the addition of a salt is accompanied by a change in the optical activity of gelatin solutions makes it seem more probable that these salts displace the equilibrium between the keto and enol forms of the protein molecules. This explanation does not exclude the possibility of a combination of a neutral salt with an adjacent nitrogen atom as suggested by Hardy. One might conceive that enolization is influenced by the attachment of a salt to the nitrogen atom adjacent to a keto group, for example:





SUMMARY AND CONCLUSIONS

It has been found in a previous investigation by the author that a neutral salt added to a solution of ash and electrolyte-free gelatin increases the hydrogen ion concentration of such a gelatin solution. It was found in the present investigation that a neutral salt at the same time reduces the optical activity of a gelatin solution while small amounts of HCl do not. These combined findings give evidence in favor of the conclusion that a neutral salt displaces the keto-enol equilibrium of a protein, decreasing the number of asymmetric carbon atoms and increasing the number of OH groups. The relative activity of various neutral salts and of small amounts of HCl on the optical activity of 2 per cent gelatin solutions has been noted.

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HEXOSEMONOPHOSPHATES

SYNTHETIC ROBISON ESTER

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We have previously described¹ the synthesis of a glucosemonophosphate, to which we assigned the structure of glucose-6-phosphate, which was similar in most properties, including enzymic fermentation, to the Robison monophosphate which occurs in enzymic fermentations. Later we showed² that the acids prepared from the Robison ester and our ester were similar, and that the lactone formation from them followed the same course. Our ester differed from the Robison only in the melting point of its osazone and in the rotation of the barium salt. Robison has now shown³ that the melting point of the osazone of his ester was incorrectly reported, the osazone being in fact identical with that from the Harden-Young and Neuberg esters which is what we had found for the osazone of our synthetic ester.

Purification of our earlier products had been found difficult because of a highly colored impurity which contaminated the brucine salt and which was not removed on further crystallization. We now find that by avoiding the use of charcoal this substance is present in much smaller amount and can be removed by recrystallization. A portion of brucine salt crystallized three times from 90 per cent methyl alcohol, as used by Robison for his ester, on reconversion to the barium salt, gave a product with a rotation of $[\alpha]_{5461}^{25} = +20.2^\circ$. Robison found for his most highly purified specimen a rotation of $+21^\circ$.

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² Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **91**, 751 (1931).

³ Robison, R., and King, E. J., *J. Soc. Chem. Ind., Chem. and Ind.*, **50**, 156 (1931).

For further confirmation of the structure of glucose- and fructose-6-phosphates we have attempted the synthesis of glucose-6-phosphate from 1,2,3,4-tetracetyl-*D*-glucose which presumably has only position 6 open.⁴ Phosphorylation of this product has been already performed by Helferich and du Mont⁵ but their method of isolation led to the securing of tri(1,2,3,4-tetracetyl glucose)-6-phosphate and tri-glucose-6-phosphate which were of no interest to us. We modified the methods of phosphorylation and isolation, and did in fact obtain some material possessing the properties of a monophosphate. Moreover, this product gave in good yield, the same phosphosazone as that from the Harden-Young ester. However, the yield of monophosphate from the tetracetyl glucose was very low, and the reaction was peculiar in that an unusually large proportion of barium monophosphate was formed along with the barium salt of the ester. In this connection it must be remembered that the 1,2,3,4-tetracetyl glucose can be isomerized⁶ with very mild alkaline treatment to a tetracetate whose structure is not yet certain.⁷ For these reasons this method of approach cannot, for the present, be considered to have yielded a conclusive confirmation of the view that the Robison ester is glucose-6-phosphate.

Our intention is to prepare next the 1,2,3,5-isodiacetone of Ohle, which has only position 6 open,⁸ and to phosphorylate it to secure a glucose-6-phosphate, the structure of which will be certain.

In the meantime, it seems certain that our synthetic product is the same substance as the naturally occurring aldosemonophosphate or Robison ester. Where this ester is needed for biological studies, the synthetic product is apt to be more easily prepared than the natural, and for many purposes the crude synthetic barium salt could be used without further purification.

⁴ Helferich, B., and Klein, W., *Ann. Chem.*, **449**, 219 (1926).

⁵ Helferich, B., and du Mont, H., *Z. physiol. Chem.*, **181**, 300 (1929).

⁶ Helferich, B., and Klein, W., *Ann. Chem.*, **449**, 219 (1926); **455**, 173 (1927).
Helferich, B., and Müller, A., *Ber. chem. Ges.*, **63**, 2142 (1930).

⁷ Haworth, W. N., Hirst, E. L., and Teece, E. G., *J. Chem. Soc.*, 1405 (1930).

⁸ Ohle, H., and von Vargha, L., *Ber. chem. Ges.*, **62**, 2425 (1929).

EXPERIMENTAL

Diacetone Glucose—The method used by Levene and Meyer⁹ for diacetone galactose has been modified somewhat and utilized for the preparation of diacetone glucose. Although the yield is only 50 per cent of the theoretical, still from the point of view of time and expense the method is a considerable improvement.

To 2 liters of acetone (U.S.P.) add 20 cc. of sulfuric acid (D, 1.84), 250 gm. of anhydrous copper sulfate, and 250 gm. of glucose (cerelose, Corn Products Company, was used) in this sequence, and shake or stir the mixture for 24 hours. Filter with suction to remove copper sulfate and unchanged glucose, and neutralize with powdered calcium hydroxide. Refilter and decolorize with charcoal. Concentrate to a thick syrup under reduced pressure and extract repeatedly with boiling heptane. Decolorize with charcoal and allow to crystallize. Yield, 155 to 165 gm.

Monoacetone Glucose—The method of Coles, Goodhue, and Hixon¹⁰ is used with slight modification.

To 600 cc. of ethyl acetate add 6 cc. of nitric acid (D, 1.42), 3 cc. of water, and 75 gm. of diacetone glucose. Immerse in a steam bath until the mixture boils, and then keep boiling for 2 minutes. Cool in running water with stirring, filter off the monoacetone glucose, and wash with more ethyl acetate. To the washings and mother liquor add ethyl acetate to 600 cc. total, 6 cc. of water, 3 cc. of the concentrated nitric acid, and 75 gm. of diacetone glucose. Heat as before. This procedure may be repeated indefinitely and serves to decrease the amount of ethyl acetate required. After hydrolyzing the last batch of diacetone glucose, add the usual proportion of water and reheat in order to hydrolyze any unchanged diacetone glucose in the mother liquors.

In recrystallizing the monoacetone glucose from ethyl acetate it is best to add enough powdered calcium hydrate to neutralize the acid which adheres to the crude monoacetone. Add calcium hydrate and charcoal to the boiling ethyl acetate solution, filter with suction, and cool. The monoacetone is obtained as a colorless, almost pure product after one crystallization.

⁹ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **92**, 257 (1931).

¹⁰ Coles, H. W., Goodhue, L. D., and Hixon, R. M., *J. Am. Chem. Soc.*, **51**, 523 (1929).

6-Glucosephosphate from Monoacetone Glucose—The procedure is essentially that used previously; the change in time of phosphorylation increases the yield a little and gives a purer product.

The isolation is somewhat improved.

Dissolve 20 gm. of monoacetone glucose in 125 cc. of dry pyridine, cool to -35° , and add, in several portions, a solution of 8.0 cc. of distilled phosphorus oxychloride in 25 cc. of cold dry pyridine, recooling the mixture to -35° before each addition. After the oxychloride is all added, allow the solution to warm slowly to -20° and then place in an ice-salt bath at -10° to -12° for 1 hour. Add chipped ice and ice water, and make alkaline to phenolphthalein by the addition of a warm saturated solution of barium hydroxide. Without filtering, transfer to a distilling flask and remove the pyridine under reduced pressure, adding more water if necessary. Dilute to a volume of 105 cc., add 45 cc. of 5 N sulfuric acid, keep at 80° for $1\frac{1}{2}$ to 2 hours,¹¹ and then cool. Without filtering, add a suspension of precipitated silver carbonate until all the chloride is removed. The mixture should still be acid to Congo paper, and more sulfuric acid should be added if it is not. When the solution is chloride-free, filter with suction. Suspend the precipitate in 250 cc. of 0.1 N sulfuric acid, stir well, and re-filter. Saturate the filtrate with hydrogen sulfide, aerate to remove entirely the excess hydrogen sulfide, make alkaline to phenolphthalein with a warm saturated solution of barium hydroxide, and filter with suction. After concentrating under reduced pressure to about half volume, re-filter, using charcoal, and continue the concentration until the volume is about 30 cc. If turbid, re-filter with charcoal. Precipitate with an equal volume of 95 per cent alcohol, filter off the precipitate, and wash with 50 per cent alcohol. Redissolve in water. Concentrate under reduced pressure to the same volume as before, re-filter with charcoal, and reprecipitate. Wash and dry as usual. Yield, 13 to 15 gm.

The product, as thus prepared, is a white colorless powder, analyzing well for the barium salt of a hexosemonophosphate, and having an optical rotation of $[\alpha]_D^{25} = +14^{\circ}$ to $+15^{\circ}$. It gives with the greatest ease the phosphosazone which melts at $150-151^{\circ}$ and has the optical properties of the osazone of the Harden-

¹¹ Josephson, K., and Proffe, S., *Ann. Chem.*, **481**, 91 (1930).

Young ester. The sodium salt of this synthetic ester is fermented by zymine at the same rate as is the naturally occurring Robison monophosphate and the initial rate is the same as that of glucose.

For purification, the barium salt prepared as above was converted to the brucine salt and this was recrystallized three times from 90 per cent methyl alcohol. The use of charcoal at this stage had to be avoided as a colored impurity was formed which could not be removed by further treatment. The three times crystallized material analyzed for the dibrucine salt of a hexose-monophosphate.

A portion of the pure brucine salt was reconverted to the barium salt and this was precipitated, washed, and dried as usual. The analysis corresponded to the barium salt of a hexosemonophosphate.

4.145 mg. substance: 23.475 mg. ammonium phosphomolybdate (Pregl).
0.100 gm. " : 0.0573 gm. BaSO₄.

C₆H₁₁O₈PBa. Calculated. P 7.84, Ba 34.74
Found. " 8.22, " 33.72

The rotation was

$$[\alpha]_D^{25} = \frac{+ 1.29^\circ \times 100}{2 \times 3.70} = + 17.4^\circ$$

and

$$[\alpha]_{4401}^{25} = \frac{+ 1.49^\circ \times 100}{2 \times 3.70} = + 20.2^\circ$$

Robison found 21.0° for this last value on samples of the most highly purified, naturally occurring ester. The purity of the preparation above could presumably be increased by further crystallization of the brucine salt, but it is quite pure enough for most purposes.

Glucose-6-Phosphate from 1,2,3,4-Tetracetyl Glucose—1,2,3,4-tetracetyl-*D*-glucose was prepared from glucose through the triphenylmethyl derivative as described by Helferich *et al.*¹² and was phosphorylated as follows:

¹² Helferich, B., Moog, L., and Jünger, A., *Ber. chem. Ges.*, **58**, 872 (1925).
Helferich, B., and Klein, W., *Ann. Chem.*, **449**, 219 (1926).

4.8 cc. of redistilled phosphorus oxychloride (1.1 mols) were dissolved in 50 cc. of cold dry pyridine and cooled to about -30° . To this was added, in several portions, a solution of 17 gm. (1.0 mol) of the pure 1,2,3,4-tetracetyl glucose in 75 cc. of dry pyridine, the mixture being recooled to -30° before each addition. Heat was evolved and pyridine hydrochloride separated, but the solution remained water-white. After all the acetoglucose had been added, the mixture was transferred to an ice-salt mixture for 2 hours and was then recooled to -35° . A 10 per cent solution of water in pyridine was added until no further heat was evolved, the temperature being kept below -20° throughout. Ice was then added and finally ice water. The mixture was still water-white.

Barium hydroxide was now cautiously added to the above solution. The temperature was kept below 5° and the mixture was well stirred but at a pH of about 6 the mixture began to darken and a precipitate formed. The precipitate increased in amount and the solution became darker in color up to a pH of about 7. The addition of the barium hydroxide was continued until the mixture was alkaline to phenolphthalein, and it was then allowed to stand for a few minutes. During this time the alkalinity had decreased; hence more barium hydroxide was added to restore the pH to its previous value. This procedure was repeated until the mixture remained alkaline even after standing. It was then transferred to a distilling flask and concentrated under reduced pressure until all the pyridine was removed, water being added once or twice. The mixture was still alkaline to phenolphthalein and remained so even after warming to 35° for 15 minutes. The large precipitate was centrifuged off, the solution was filtered with charcoal, concentrated to a small volume, and precipitated with an equal volume of 95 per cent alcohol. After being washed with 50 per cent alcohol, the precipitate was dissolved in water, the solution was concentrated under reduced pressure, filtered with charcoal, and reprecipitated with an equal volume of alcohol as before. The solution and precipitation were repeated once more, and the product, which was perfectly white, was dried. The yield was only 3.2 gm., and the rotation was low; $[\alpha]_D^{25} = +11.7^{\circ}$.

The analysis was only fair.

3.950 mg. substance; 23.185 mg. ammonium phosphomolybdate (Pregl).

0.0860 gm. " : 0.0490 gm. BaSO₄.

C₆H₁₁O₆PBa. Calculated. P 7.84, Ba 34.74

Found. " 8.52, " 33.53

The barium was removed from 1.3 gm. of the material and the osazone was formed in the usual fashion. The yield was 0.7 gm. which is as good as that from an equal quantity of the Neuberg or Robison ester. It melted at 149–150° and there was no lowering of the melting point when mixed with an authentic sample of the Harden-Young osazone. The rotation was –53.4° in pyridine-absolute alcohol (2:3 by volume) 5 minutes after dissolving and

$$[\alpha]_D^{25} = \frac{-0.19^\circ \times 100}{0.5 \times 1.2} = -31.7^\circ \text{ (at equilibrium)}$$

Effect of Glucose-6-Phosphate on Induction Period—A sample of zymin prepared from a bottom yeast was used which exhibited an induction period of 90 minutes. Addition of hexosediphosphate to a concentration of 0.005 mol per liter entirely removed this induction and fermentation began immediately. With both synthetic glucose-6-phosphates, that from the monoacetone glucose and that from the 1,2,3,4-tetracetyl glucose, at concentrations of 0.005 mol per liter the period of induction was reduced to 45 minutes. This is of the same order of magnitude as the effect previously observed for the naturally occurring Robison ester at the same concentration.

HEXOSEMONOPHOSPHATES

GALACTOSE-6-PHOSPHATE

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Through the extensive researches of Harden and others it has been shown that accompanying the enzymic fermentation of sugars, and also the enzymic processes taking place in muscle, there are large amounts of sugar phosphates simultaneously formed, and in some cases destroyed. The function of such phosphorylations and dephosphorylations has however not been elucidated. Whether some phosphate, as yet unisolated, acts as a true intermediate in the processes, or whether in some fashion these phosphates modify or condition the cleavage of the sugars, is as yet not known.

One of the ways of elucidating the function of the phosphoric esters in fermentation is to establish whether the mere phosphorylation of an otherwise unfermentable hexose endows it with this property. For example, would galactose, which is fermented by ordinary yeasts only after suitable culturing, be fermented by the yeast enzyme, if first converted into a phosphoric ester? Inasmuch as the only glucose phosphate which is readily fermentable is that in which the substitution is in position 6, the obvious ester to test would be galactose-6-phosphate. We have therefore prepared this ester and have tested its fermentability with zymine. The result was entirely negative.

A second property possessed by certain phosphorylated sugars is the ability to diminish the period of induction which is observed with certain preparations of zymine. The unsubstituted sugars are without effect. Of the phosphoric esters the hexosediphosphate has this property to the greatest degree while the Robison (glucose-6-phosphate) and Neuberg (fructose-6-phosphate) esters

are less effective. We now find that galactose-6-phosphate is negative in this respect.

It is our intention to examine next the behavior of the galactose-phosphates with enzymes from yeasts which have the ability to ferment galactose.

The present observations then, serve to show that glucose-6-phosphate may not be simply an accidental product, but a substance functioning in the process of fermentation.

EXPERIMENTAL

Galactose-6-Phosphate from Diacetone Galactose—Diacetone galactose was prepared (by Dr. G. M. Meyer) by the procedure described by Levene and Meyer¹ and distilled under greatly reduced pressure.

It was phosphorylated as follows: 7.1 cc. of redistilled phosphorus oxychloride (1.1 mols) were dissolved in 75 cc. of cold dry pyridine and cooled to -30° . To this was added in three portions, a solution of 20 gm. (1.0 mol) of diacetone galactose in 50 cc. of dry pyridine, the mixture being recooled to -30° before each addition. After addition of all of the diacetone, the mixture was transferred to an ice-salt bath for 2 hours and was then recooled to -30° . A solution of 10 per cent of water in pyridine was added until no further heat was evolved and ice and then ice water were added. The mixture was neutralized with barium hydroxide, the pyridine was removed by distillation under reduced pressure, and the acetone groups were hydrolyzed off, sulfuric acid at 80° being used exactly as described for the phosphate prepared from monoacetone glucose.² The subsequent treatment was likewise the same, the halide being removed with silver carbonate and the excess sulfuric acid with barium hydroxide. After concentration under reduced pressure to a small volume, the material was precipitated by the addition of an equal volume of 95 per cent alcohol, was washed with 50 per cent alcohol, redissolved, concentrated, precipitated, washed, and dried as usual. The yield was 12 to 13 gm.

¹ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **92**, 257 (1931).

² Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **92**, 757 (1931).

TABLE I
*Fermentation by Zymin of Galactose-6-Phosphate, Glucose-6-Phosphate,
 and Glucose*

Time min.	Cc. CO ₂ per gm. zymin			
	Blank	Galactose-6-phosphate	Glucose-6-phosphate	Glucose + sodium phosphate
4				
9	0.14	0.13	0.65	0.45
15	0.28	0.44	2.24	2.88
30	1.11	1.15	3.93	5.63
45	1.85	1.90	5.24	7.08
60	2.26	2.38	5.48	8.38
90	2.68	2.83	6.64	10.77
170	2.96	3.05	8.93	16.84

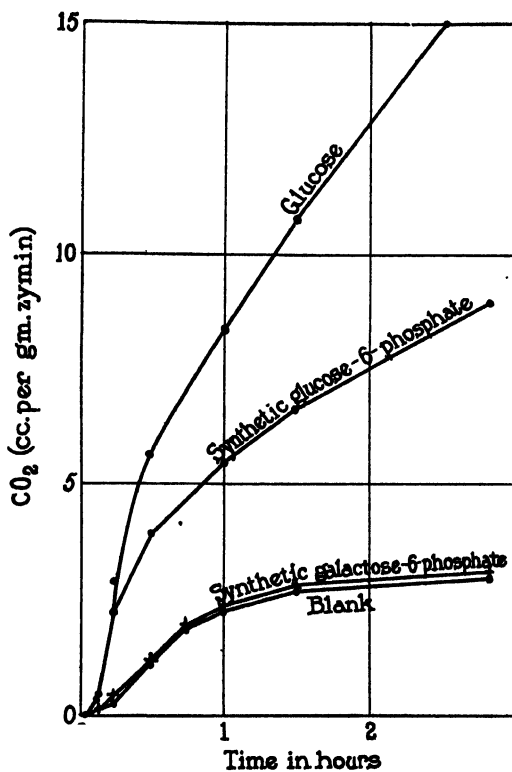


FIG. 1

The analysis corresponded to the barium salt of a hexosemono-phosphate.

4.555 mg. substance: 24.595 mg. ammonium phosphomolybdate (Pregl).

0.0909 gm. " : 0.0528 gm. BaSO₄.

C₆H₁₁O₅PBa. Calculated. P 7.84, Ba 34.74

Found. " 7.84, " 34.18

and the rotation was

$$[\alpha]_D^{25} = \frac{+1.96^\circ \times 100}{2 \times 4.0} = +24.5^\circ$$

Fermentations—The apparatus and technique were identical with those previously described.³ The esters were used in the form of their sodium salts and at concentrations of 0.06 mol per liter. Where glucose was used its concentration was 10 per cent. The induction period exhibited by the zymin was removed by adding hexosediphosphate to the extent of 0.005 mol per liter to each tube. Toluene was added in all cases. The data are given in Table I and are plotted in Fig. 1.

Effect of Galactose-6-Phosphate on Induction Period—A sample of zymin prepared from a bottom yeast was used which exhibited an induction period of 90 minutes. With the esters at a concentration of 0.005 mol per liter it was found that the induction period was entirely removed by hexosediphosphate and was reduced to 45 minutes by the synthetic glucose-6-phosphate. The galactose-6-phosphate on the other hand was slightly inhibiting and fermentation did not begin for 110 minutes.

³ Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **79**, 621 (1928).
Raymond, A. L., *J. Biol. Chem.*, **83**, 611 (1929).

γ -GLUCOSIDE OF 3-METHYL-*d*-GLUCOSE

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INTRODUCTION

The present communication presents part of the work on the general problem of the influence of partial substitution on the rate of glucoside formation and on the ring structures of the glucosides that are formed. The specific interest of 3-methyl-*d*-glucose lies in the fact that this sugar is incapable of forming a propyleneoxidic glucoside. Thus, should this sugar form a γ -glucoside under the same conditions as unsubstituted *d*-glucose, then the propyleneoxidic structure is excluded for the γ -glucosides. Also, if the general conduct and the rate of glucoside formation of the two γ -glucosides are identical it will be warranted to conclude that the ring structure of the two γ -glucosides is identical. Actually, 3-methyl-*d*-glucose formed both a γ and a normal glucoside, and, moreover, the rate of formation of the γ form was essentially the same as that of the γ -glucoside from *d*-glucose. This evidence alone might suffice to exclude the propyleneoxidic structure for the γ -methylglucosides, but for greater rigor it was deemed desirable to compare the tetramethyl- γ -methylglucosides obtained from *d*-glucose and from 3-methyl-*d*-glucose. As will be seen from the experimental part these two γ -glucosides were identical.

To exclude very definitely the possibility of the propyleneoxidic structure for the γ -glucosides, the formation of glucosides of a sugar methylated in position (4) should be studied since in this case the formation of only a pyranose glucoside should be observed if the propyleneoxidic structure is to be excluded for the γ form. Work in this direction is now in progress.

EXPERIMENTAL

Rate of Glucoside Formation—The glucoside formation of glucose and 3-methylglucose was determined in methanol by following the rotation of the solutions. The alcoholic solutions contained 5.0 gm. of dry hydrogen chloride and 0.0344 mol of sugar per liter. Experiments were made at room temperature (23–25°) and at 76°. At the lower temperature, the solutions were placed in 4 dm. polariscope tubes immediately after their preparation and the rotations were measured at various intervals. At 76°, the solutions

TABLE I
Observed Rotations during Glucoside Formation

25°			76°		
Time	Rotation α_D^{25} in 4 dm. tube		Time	Rotation α_D^{25} in 2 dm. tube	
	Glucose	3-Methyl-glucose		Glucose	3-Methyl-glucose
<i>hrs.</i>	<i>degrees</i>	<i>degrees</i>	<i>hrs.</i>	<i>degrees</i>	<i>degrees</i>
0.08	+1.65	+1.79	0.00	+0.88	+0.90
0.67	1.19	1.23	0.08	0.06	−0.54
1.50	0.88	0.79	0.17	0.16	−0.14
2.25	0.63	0.41	0.33	0.31	−0.04
3.00	0.45	+0.12	1.00	0.80	+0.50
5.58	+0.04	−0.54	2.00	1.14	0.89
11.0	−0.18	−0.85	5.00	1.33	1.23
21.0	−0.09	−0.84	10.0	1.39	1.41
27.0	−0.01	−0.81	20.0	1.41	1.54
54.0	+0.19	−0.51	30.0	1.42	1.55
			50.0	+1.41	+1.57

were mixed in the cold (0°) and small samples were sealed in glass tubes. These tubes were then placed in a bath of boiling carbon tetrachloride (76°) for given intervals of time after which they were quickly cooled to room temperature. The rotations were measured in a 2 dm. tube at 23–25° with sodium D light. They are given in Table I. Specific rotations calculated from these data are plotted in Fig. 1.

3-Methyl- γ -Methylglucoside—35 gm. of 3-methylglucose were dissolved in 175 cc. of anhydrous methanol containing 0.87 gm. of dry hydrogen chloride. The solution was heated to boiling and was

refluxed for exactly 20 minutes. It was then cooled quickly in ice water. During this heating the rotation of the solution changed from the initial value of $[\alpha]_D^{25} = +67^\circ$ to a value of $[\alpha]_D^{25} = -3.2^\circ$. The solution was next treated with silver carbonate to remove the hydrogen chloride, with hydrogen sulfide to remove the excess silver, and was then evaporated to a thick syrup

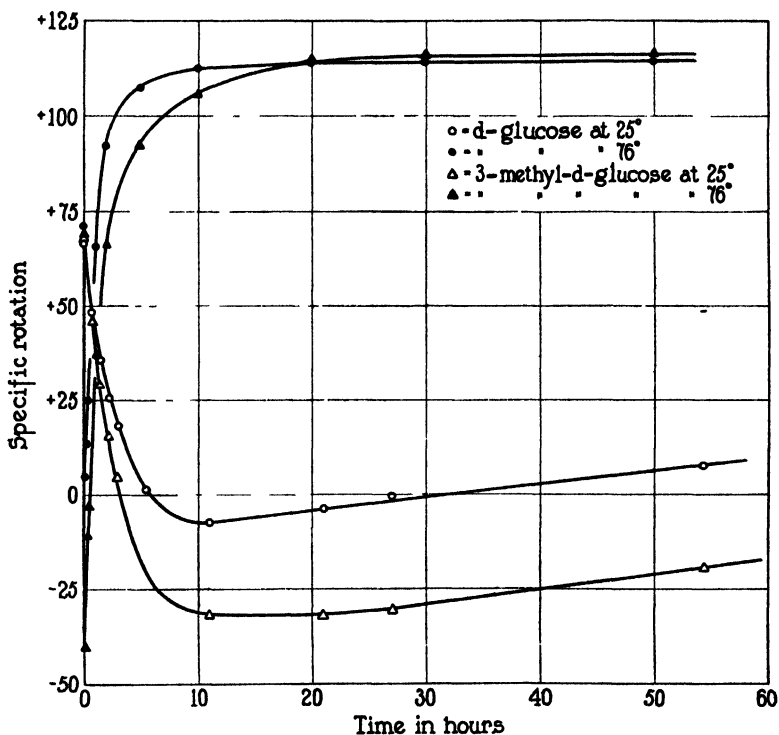


FIG. 1. Glucoside formation

under diminished pressure. The bath temperature during this concentration was kept below 35° to avoid any destruction of the γ -glucoside. The yield of practically colorless syrup was 40 gm. (theoretical 38 gm. for 3-methylmethylglucoside). The syrup was extracted with a total of 900 cc. of dry neutral ethyl acetate leaving a residue of 11.9 gm. The ethyl acetate extract was concentrated at a low temperature to a thick syrup. Weight, 28 gm.

The analysis indicated 6 per cent free sugar, 77 per cent 3-methyl- γ -methylglucoside,¹ and 17 per cent normal 3-methylmethylglucoside (by difference).

Tetramethyl- γ -Methylglucoside—The 3-methyl- γ -methylglucoside was methylated with 280 gm. of methyl iodide and 70 gm. of silver oxide. The methylating mixture was stirred continuously during the process which required 5 hours. The methylated product was extracted with hot chloroform, the extract was dried with anhydrous sodium sulfate, and the chloroform removed under diminished pressure. The product, 27 gm., was remethylated and isolated as before, giving 25 gm. of material. This was distilled at 0.3 to 0.8 mm. giving 23 gm. of product boiling at 95–107°, whose analysis corresponded to 3 per cent free sugar and 79 per cent tetramethyl- γ -methylglucoside. The rotation was

$$[\alpha]_D^{20} = \frac{+0.60^\circ \times 100}{2 \times 4.14} = +7.3^\circ \text{ (in methanol)}$$

20 gm. of the once distilled methylated material were distilled three times at 0.4 to 0.8 mm., the last portion of the distillate being rejected each time. The boiling point of the final product, weight 14 gm., was 95–105°. The analysis indicated 1.3 per cent free sugar and 81 per cent tetramethyl- γ -methylglucoside. The rotation was

$$[\alpha]_D^{20} = \frac{+0.77^\circ \times 100}{2 \times 4.09} = +9.4^\circ \text{ (in methanol)}$$

$$[\alpha]_D^{20} = \frac{+0.72^\circ \times 100}{2 \times 4.07} = +8.8^\circ \text{ (in water)}^2$$

and the refractive index was n_D^{20} , 1.4426; n_D^{25} , 1.4406. It had the following composition.

6.265 mg. substance: 12.109 mg. CO₂ and 4.920 mg. H₂O.

4.121 " " : 19.250 " AgI.

C ₁₁ H ₂₂ O ₆ .	Calculated.	C 52.76, H 8.86, OCH ₃ 62.03
250.2	Found.	" 52.70, " 8.70, " 61.66

¹ This was determined by hypoiodite titration (Willstätter and Schudel method) for aldose before and after hydrolysis with 0.1 N hydrochloric acid for 10 minutes at 100°.

² This final distilled product dissolved in water giving a clear solution. The original undistilled methylated product and even twice distilled samples gave quite opaque water solutions whose rotations could not be easily measured.

The combined residues from the three distillations consisted of 7 per cent free sugar and 80 per cent γ -glucoside. The rotation was

$$[\alpha]_D^{25} = \frac{+1.75^\circ \times 100}{2 \times 4.364} = +20.1^\circ \text{ (in methanol)}$$

and the refractive index was n_D^{25} , 1.4440.

Tetramethyl- γ -Glucose—2.52 gm. of the purified tetramethyl- γ -methylglucoside (81 per cent γ form and 18 per cent normal form) were dissolved in 65 cc. of 0.1 N hydrochloric acid, the solution was heated on the steam bath for exactly 10 minutes, and was then cooled in ice water. A hypiodite titration for aldose indicated 75 per cent hydrolysis of the total glucoside.³ During the hydrolysis the rotation changed from $[\alpha]_D^{22} = +8.3^\circ$ to $[\alpha]_D^{22} = -8.8^\circ$. This tetramethyl- γ -glucose was oxidized directly without isolation as indicated below.

2,3,5,6-Tetramethylgluconic Acid-Barium Salt—The solution of tetramethyl- γ -glucose was added to 100 cc. of a solution containing 2.6 gm. of iodine and 5.0 gm. of barium iodide. To this were added 80 cc. of 0.53 N barium hydroxide over a period of 20 minutes during which time the solution was continuously stirred. The mixture was then allowed to stand for 20 minutes after which it was acidified with 5 cc. of 5 N sulfuric acid. The liberated iodine was reduced with sulfur dioxide to iodide ion and the total halide was precipitated with a slight excess of silver sulfate.⁴ The combined precipitate was filtered off and the filtrate was treated with hydrogen sulfide. It was then aerated and the silver sulfide removed by filtration. Barium hydroxide was now added until the mixture was slightly alkaline to phenolphthalein, the barium sulfate was centrifuged off and the solution was concentrated under diminished pressure. During this concentration some barium carbonate separated out. This, as well as a small amount of

³ Hydrolysis of a small sample for 25 hours at 100° with 0.1 N hydrochloric acid gave a solution which by titration indicated 98 per cent hydrolysis to aldose. The rotation was $[\alpha]_D^{25} = +1.2^\circ$.

⁴ The silver sulfate was freshly prepared from the calculated quantity of 5 N sulfuric acid by adding silver carbonate until a slight excess of the latter was present. The entire mixture was then added to the solution containing the halide.

barium sulfate not removed by the preceding centrifuging was easily filtered off after the solution had been reduced to 100 cc. volume. The clear solution was further concentrated to a syrup. This was taken up in acetone from which a white amorphous precipitate was thrown out by the careful addition of anhydrous ether. The precipitate was removed, the filtrate reevaporated, and a second crop of material obtained as before. In all, three crops were obtained which weighed 2.5 gm. This material was redissolved in a small amount of water, in which it was exceedingly soluble, acetone was added, and the material was reprecipitated with anhydrous ether. The reprecipitated material contained 7.0 per cent of moisture. Its analysis corresponded to a barium salt of tetramethylgluconic acid. A hypiodite titration indicated only a trace of aldose.

6.811 mg. substance: 2.561 mg. BaSO₄.

4.290 " " : 13.012 " AgI.

C ₂₀ H ₃₈ O ₁₄ Ba.	Calculated.	Ba 21.48,	OCH ₃ 38.80
639.7	Found.	" 22.12,	" 40.22

It had the following rotation.

$$[\alpha]_D^{25} = \frac{+1.23^\circ \times 100}{2 \times 1.852} : +33.2^\circ \text{ (in water)}$$

The yield of barium salt, 2.32 gm. (dry) was 96 per cent of the theory on the basis of 75 per cent hydrolysis of the original tetramethylmethylglucoside.

0.2028 gm. of the barium salt (dry) was dissolved in 3 cc. of water, 6.50 cc. of 0.1 N hydrochloric acid were added, and the solution was made up to exactly 10 cc. This amount of acid is equivalent to the barium present. The rotation of the solution was followed at room temperature (23–25°) for 67 hours, and the equilibrium rotation was then found by warming the solution for short intervals of time. The rotations are given in Table II. The time required to reach half equilibrium rotation is at least 150 hours at 25° which is compatible with the rate of formation of other γ -lactones as indicated by the results of Levene and Simms⁵ and of Carter, Haworth, and Robinson.⁶ It is entirely different

⁵ Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, **68**, 737 (1926).

⁶ Carter, S. R., Haworth, W. N., and Robinson, R. A., *J. Chem. Soc.*, 2125 (1930).

from the rate of formation of the δ -lactones as observed by these investigators.

0.5 gm. of the barium salt was dissolved in 12 cc. of water and the calculated equivalent of sulfuric acid (7.53 cc. of 0.19 N) was added. The solution was warmed slightly to coagulate the barium sulfate which was filtered off with the aid of charcoal. The clear filtrate was evaporated to dryness and was heated for a short time at 100° under diminished pressure. The lactone was taken up in 10 cc. of ether and a solution of 0.158 gm. of phenylhydrazine in 2.9 cc. of ether was added. The solvent was evaporated off, as was a

TABLE II
Lactone Formation at 25°

Time	Rotation α_D^{25} in 2 dm tube	$[\alpha]_D^{25}$
<i>hrs.</i>	<i>degrees</i>	<i>degrees</i>
0.0	+0.69	+21.6*
0.3	0.70	21.9
1.0	0.71	22.2
2.0	0.71	22.2
17.5	0.74	23.2
26.0	0.76	23.8
50.0	0.80	25.0
67.0	0.83	26.0
∞	+1.20	+37.6

* The rotation of the free acid is slightly lower than that reported by Drew, Goodyear, and Haworth (Drew, H. D. K., Goodyear, E. H., and Haworth, W. N., *J. Chem. Soc.*, 1237 (1927)) but the difference is not sufficient to affect appreciably the calculation of the rate.

second addition of 15 cc. more. To the syrupy residue were added 10 cc. of benzene. From this solvent a flocculent precipitate separated on the addition of ether. The solution was left overnight in the ice box after sufficient ether had been added to bring down a slight precipitate at the boiling point of the mixture. Practically colorless needles separated. After once recrystallizing from benzene they melted at 130.5–132.5° (corrected). A second recrystallization from ether-benzene raised the melting point to 135.3–135.8° (corrected). Haworth, Long, and Plant⁷ report a

⁷ Haworth, W. N., Long, C. W., and Plant, J. H. G., *J. Chem. Soc.*, 2809 (1927).

melting point of 135–136° for the phenylhydrazide of 2,3,5,6-tetramethylgluconic acid. The melting point of the 2,3,4,6-tetramethyl derivative is 115° and its rotation is $[\alpha]_D = +42.1^\circ$ (in ethanol).

The analysis corresponded to the phenylhydrazide of a tetramethylgluconic acid.

4.444 mg. substance: 9.144 mg. CO₂ and 3.040 mg. H₂O.

4.390 " " : 0.318 cc. N (755 mm. and 25°).

6.206 " " : 17.111 mg. AgI.

C ₁₈ H ₂₈ O ₆ N.	Calculated.	C 56.11,	H 7.66,	N 8.19,	OCH ₃ 36.27
342.2	Found.	" 56.11,	" 7.65,	" 8.24,	" 36.39

The rotation was

$$[\alpha]_D^{21} = \frac{+0.13^\circ \times 100}{2 \times 0.112} = +58^\circ \text{ (in ethanol)}$$

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